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BIOMARKERS OF EXPOSURE TO TOXIC SUBSTANCES

Volume V: Biomarker Pre-validation Studies
Pre-validation of Urine and Serum Biomarkers Indicative of
Subclinical Kidney Damage in a Nephrotoxin Model

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14. ABSTRACT
Once biomarkers have been identified using Omics technologies, assays must be developed and the markers pre-validated in both the test model samples and in other relevant kidney injury models. To initiate work to examine biomarkers to subclinical kidney injury, two separate efforts were conducted. First, extensive literature searches were conducted to compile a list of potential kidney injury markers identified in other disease or injury models, either human or animal. Secondly, unique markers identified through RHPB's Omics efforts were examined. In both cases, urinary protein markers were analyzed for sensitivity and selectivity for injury levels, regional damage, temporal response and dose response following nephrotoxin exposure. Two experimental nephrotoxins were interrogated, D-serine and puromycin, each previously demonstrated to degrade a specific kidney segment (proximal tubule and glomerulus, respectively). In this study a total of seventeen protein biomarkers were evaluated, with ten journal-based markers pre-validated and two unique RHPB markers partially pre-validated. Two other RHPB markers were examined and pre-validation was partially completed. The current panel set of 11 biomarkers is indicative of very low level subclinical kidney damage/dysfunction with a response range in the urine between the earliest time point of 12 hours to a maximum of 96 hours post-toxin exposure.

15. SUBJECT TERMS
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i

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TABLE OF CONTENTS

Section   Page

LIST OF FIGURES

Figure **Page**

LIST OF TABLES

FORWARD

This research program is documented in a final technical report comprised of five volumes. Volume I provides a global overview of the entire effort. Volumes II-IV provide the technical details of the three approaches (genomics, proteomics, and metabonomics) used to identify the relevant biomarkers of toxic effects. Volume V describes the effort to perform pre-validation of the identified biomarkers.

Figure **1** shows this technical report structure.
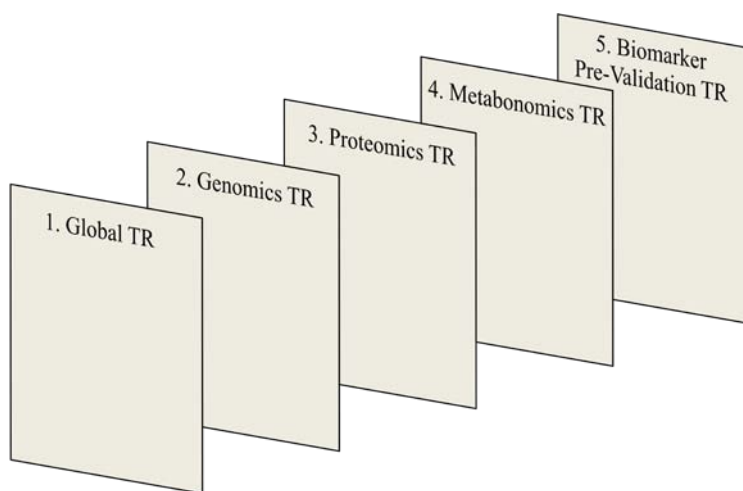
Figure 1: Technical Report Volume Order

Volume I contains the experimental design, explains how the needs of the warfighter led to conducting this research effort, the reasoning behind the specific analysis method and biomarker selections, and the manner in which the specimens were collected. The sample analysis is captured in Volumes II-IV (Genomics, Proteomics, and Metabonomics). The three analytical and investigational approaches were conducted in parallel and fed data into Volume V (Biomarker Pre-validation) as depicted in Figure 2.
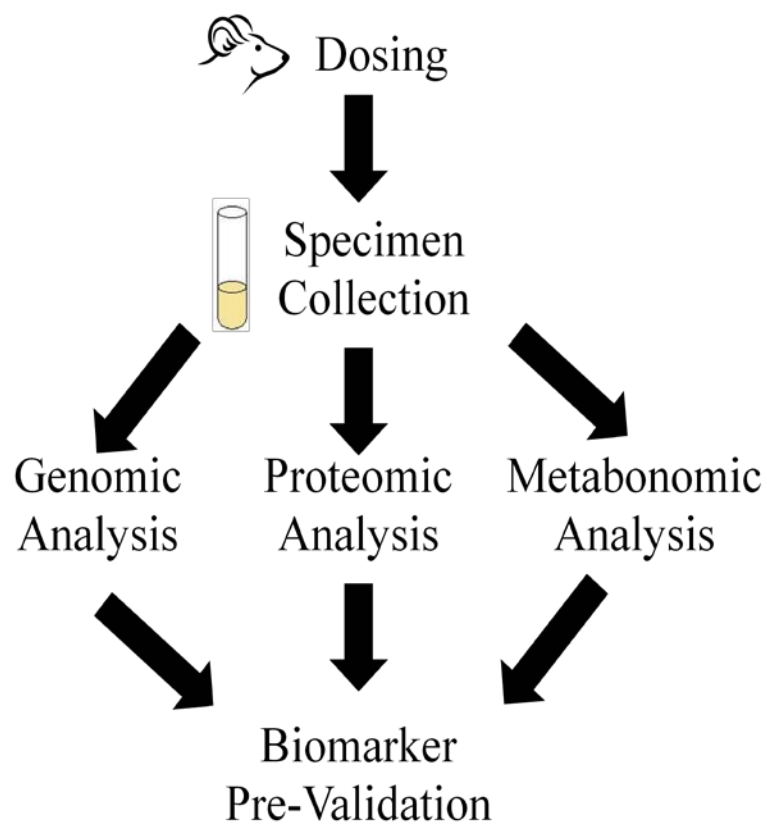
Figure 2: Work Unit Investigational Overview

Over 80 Department of Defense civilians, contractors, and military contributed in the research spanning five years.

PREFACE

This research was accomplished at the Applied Biotechnology Branch, Human Effectiveness Directorate of the 711th Human Performance Wing (711 HPW/RHPB) of the Air Force Research Laboratory, Wright-Patterson AFB, OH, under Dr. John J. Schlager, Branch Chief. This technical report was written for AFRL Work Unit 7184D405.

All studies involving animals were approved by the Wright-Patterson Institutional Animal Care and Use Committee, and were conducted in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International, in accordance with the *Guide for the Care and Use of Laboratory Animals*, National Research Council (1996). Studies were conducted under approved Air Force Research Laboratory Institutional Animal Care and Use Committee Protocol F-WA-2003-0074-A.

Some patentable information has been redacted from the DTIC open report. For full information, contact authors.

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SUMMARY

Once biomarkers have been identified using Omics technologies, assays must be developed and the markers pre-validated in both the test model samples and in other relevant kidney injury models. To initiate work to examine biomarkers to subclinical kidney injury, two separate efforts were conducted. First, extensive literature searches were conducted to compile a list of potential kidney injury markers identified in other disease or injury models, either human or animal. Secondly, unique markers identified through RHPB's Omics efforts were examined. In both cases, urinary protein markers were analyzed for sensitivity and selectivity for injury levels, regional damage, temporal response and dose response following nephrotoxin exposure. Two experimental nephrotoxins were interrogated, D-serine and puromycin, each previously demonstrated to degrade a specific kidney segment (proximal tubule and glomerulus, respectively). In this study a total of sixteen protein biomarkers were evaluated, with eleven journal-based markers pre-validated and two unique RHPB markers partially pre-validated. Two other RHPB markers were examined and pre-validation was partially completed. The current panel set of 11 biomarkers is indicative of very low level subclinical kidney damage/dysfunction with a response range in the urine between the earliest time point of 12 hours to a maximum of 96 hours post-toxin exposure.

1. INTRODUCTION

Use of Biomarker tests for renal injury

Despite well-known limitations, currently the most widely used biomarkers for the early diagnosis of CKD and AKI are serum/urine creatinine and blood urea nitrogen (Price, 2000). While highly monitored, serum/urine creatinine and blood urea nitrogen are poor biomarkers of renal status due to inherent characteristics of expression - the serum levels in part depend on the generation by the muscle (large or small body mass). In addition, both creatinine and blood urea nitrogen are affected by filtration renal mechanism, in that creatinine is secreted while urea nitrogen is reabsorbed by the renal tubules (Star, 1998). It has also been show that creatinine, serum creatinine, and urea appear days after an acute kidney injury. This time lag is too late to initiate any type of intervention to limit kidney cellular injury, as up to 70% of the kidney function may be lost before the damage is discovered (Fernholm, 2008). In addition, endogenous substances can interfere with the test analysis. For these reasons it is imperative that new biomarkers for earlier detection of renal injury are identified and validated. New markers, in both blood and urine, will allow the quatitation of kidney injury at levels at which interdiction can reverse decrement, either by recognition of toxin and/or removal from area of toxin exposure.

Nephrotoxin modeling of Renal Injury

The use of well characterized nephrotoxins to target specific kidney segments has been published in a number of studies (Ferguson et al., 2008; Wang et al., 2008; Amin et al., 2004; Boudonck et al., 2009). Many chemicals, antibiotics, and occupational toxins have been identified as initiating renal injury at the kidney (Figure 3). Many of these trigger significant injury in a single dose, and thus are useful for modeling acute renal injury. Others require several doses over longer periods of time, and are often used to model chronic exposures – especially useful for examining decrement due to long term use of medicines whose use requires careful monitoring of renal function, such as NSAIDS (Lafrance et al., 2009). For RHPB biomarker discovery, several nephrotoxins were used in rat studies to produce injury at different kidney segments (DelRaso et al., Sep 2009) and both urine and serum were examined for potential biomarkers.
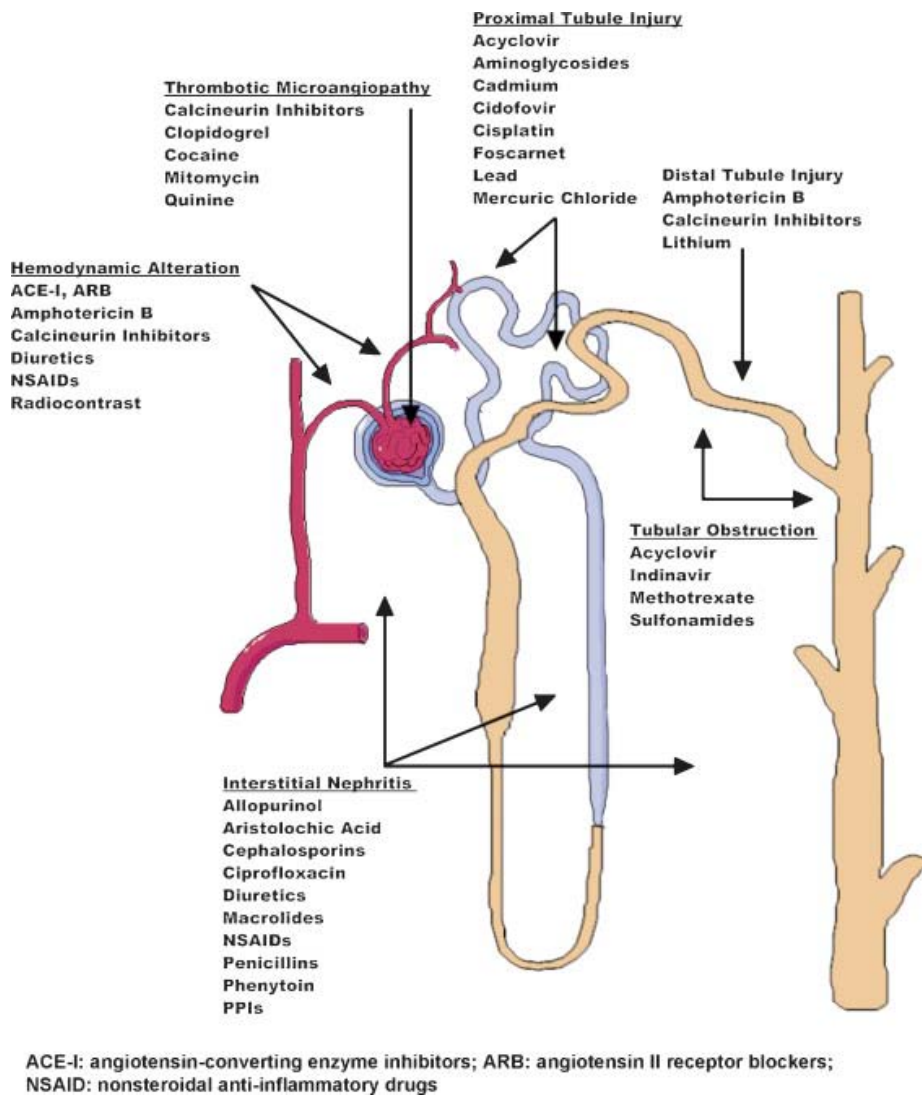
Figure 3: Known nephrotoxins and their respective target segments in the kidney
Taken from: Toxicology 245:182-193 Biomarkers of nephrotoxic acute kidney injury
MA Ferguson, VS Vaidyaand J V Bonventre.

Biomarker Test Matrices

While serum or plasma may be an attractive test matrix for the clinic, the use of urine for on-site field testing in real time is appealing for several reasons. First, kidney biomarker expression changes occurring after nephrotoxin exposure would be expected to have a faster response time in urine than that found in the blood (Otu et al., 2007). Second, proteomic analyses of urine is less complicated due to the lack of high concentration serum proteins which may mask analyses for low level protein changes without proper removal (Pisitkun et al., 2004, Seam et al., 2007). Third, the collection and testing of urine is far less invasive than that of blood (serum), and would require little processing prior to testing, as well as permitting the concept of daily collection and monitoring a viable option. It has been seen, even during this study, that urine salts can and do have an effect on certain ELISA tests. In addition, some protein biomarkers are more susceptible to proteolysis while in a urine test matrix. However, these

2

characteristics are not universal among the biomarkers examined, and such data can and should be used for decisions make on the exclusion/inclusion of any marker onto a biomarker panel set.

Biomarker Pre-validation

Once data from disease/toxin models using 'integrated Omics' have been obtained and bioinformatic analyses have identified potentially novel biomarkers, further quantitative testing must be completed in both animal and human model systems. These pre-validation and validation studies for proteins are usually accomplished by the development of ELISAs (enzyme-linked immunosorbent assay) to allow quantitation of the biomarker presumed to be indicative of a given condition, dose, or time. The ELISA is an antibody-based assay in which a capture element (the primary antibody) is bound to wells of a micro titer plate (usually 96 well) and which selectively binds a defined epitope of a given protein (biomarker) in sample solutions. The primary antibody is usually developed by the use of purified biomarker protein or a chemically synthesized peptide segment based on its protein sequence. This material (antigen), when injected into an animal, elicits an immunological response against the protein by means of stimulation of the host B-lymphocytes to produce IgG immunoglobins against the protein material. This response permits the purification of the primary antibody using serum from the antigen- injected animal which will specifically and selectively bind to the antigen used. A secondary antibody, which binds the same protein molecule but usually at a different region (epitope), is used for detection. These 'detection' antibodies have chemicals or enzymes conjugated to the antibody or are a target for a third antibody/substrate. There are several different types of ELISAs but all, with careful preparation and selection, should detect a biomarker at ng to pg per ml in fluids such as urine and serum.

RNA isolated from peripheral blood mononuclear cells (PBMCs) or tissue may also be utilized as biomarkers using DNA based capture elements to develop transcriptomic biomarker assays. In essence, with some technical changes, the same relative concept as the ELISA for proteins with the hybridization of strands providing selectivity rather than protein/antibody binding. Such assays, used as a panel, can be as sensitive and selective as proteins to elucidate a given condition/injury as protein based assays. In addition, Real-Time quantitative PCR (RT-qPCR) can also be used to determine gene expression at any given time (Nygaard et al., 2009). Therefore, research into the use of gene expression is a valid approach for development of genomic biomarkers for nephrotoxicity (Wang et al., 2008). However, the isolation of RNA from PBMCs and the sensitivity of RNA to degradation during isolation from sample render it unlikely to develop into a fieldable, self-contained assay system within the near future. Indeed, a fully validated clinical diagnosis test using RNA was only initiated in April 2008 (Szafranska et al., 2008).

Biomarker Validation

It is important to recognize that until complete and definitive human validation studies have been accomplished, the biomarker can only be considered a 'potential' or 'pre-validated' marker. Biomarker validation studies are in themselves an expensive and extensive process involving standardization of the assay/reagents and statistical analysis of test data for such parameters as sensitivity, specificity, and testing limits (analytical validation). Final validation of

the clinical ELISA involves confirmation of the utility and reproducibility of the test in human clinical trials (clinical validation) (Mandrekar et al., 2009).

Journal-based Biomarker Pre-validation Using Nephrotoxin Models

To initiate the examination of potential subclinical kidney injury biomarkers, an extensive literature search was conducted to identify previously published markers which may detect the low level kidney injury produced in our nephrotoxin model system. Searches were limited to markers indicative of acute or chronic kidney injury. Biomarkers found in association with other nephrotoxin models, such as puromycin, were ranked high on the list to examine using the D-serine nephrotoxin model. This search revealed a number of significant protein biomarkers which have been examined in the urine, serum, or both (Trof et al., 2006; Amin et al., 2004; Perco et al., 2006; Hewitt et al., 2004; Amin et al., 2004). In addition, the searches revealed a large list of candidate proteins to examine (Perco et al., 2006; Hewitt et al., 2004; Trof et al., 2006), and a further down-select was necessary. Markers were eliminated which were expressed in response to other physiological conditions, usually cancer. Of the remaining proteins, the availability of commercial ELISA tests for the specific biomarker was acertained. In many cases, a human ELISA was available. In those cases, validation tests were performed to determine if the ELISA would react to the rat homolog. In some cases, the test was validated for use in serum alone. For those assays, validation tests were conducted on the ELISA to determine its performance in the test matrix, urine.

RHPB Biomarker Pre-validation Using Nephrotoxin Models

New unpublished biomarkers (mRNA, metabolite, and protein) were the resultant products generated from the intensive RHPB-based analyses of blood and urine from the rat nephrotoxin models (discussed in Chan et al., 2009; Shiyanov et al., 2009; DelRaso et al., Oct 2009). All three Omics technologies produced unique marker lists which were examined in detail to create a sub-list of biomarkers to study as potential biomarkers for identifying subclinical, early renal injury. Due to time and funding limitations, only a small portion of proteins were submitted to pre-validation studies (hornerin, group specific component, and p-selectin). These three were chosen based on fold expression changes as well as published data.

DBBM Biomonitor Device Development

To enable the field usage of any biomarker identified either in this study or other research, new technologies in microfluidics and microelectronics must be utilized to create a small, handheld unit capable of collecting, preparing, and analyzing biomarkers. While the use of the clinical ELISA is well established, cutting edge detection elements and microfluidic fabrication are being used to develop small, point-of-care devices that may revolutionize both clinical and home care. There are numerous approaches for detection in these prototypes, ranging from Raman spectrophotometry, microcantilevers (Wu et al., 2001), ellipsometry (Ostroff et al., 1998), to hydragels (Carrigan et al., 2005). All have both pros and cons for use, but all center on the use of antibodies as capture elements and thus are subject to inherent stability limitations. There are currently several POCD (point-of-care devices) currently on the market, and the strong need for home POCDs is predicted to generate revenues of over $2.4 billion by 2012 in the US

alone (Newswire Today, 2006). However, a DoD field test unit does have separate unit requirements which complicate the development of a DBBM (deployable biomarker-based monitor) compared to that of a POCD. First, it must be small and lightweight, have a minimal footprint, require no refrigeration, and run on batteries. Sample insertion, assays and readings must be done by non-medical personnel, with data and sample storage for possible further analysis (Figure 4). Such technical requirements place the DBBM as a much more stringent technical challenge than POCDs.

While the purpose of this research was the identification and pre-validation of new biomarkers to subclinical renal damage, such efforts would be of little value for on-site real-time evaluations without significant efforts in developing the DBBM. Such concurrent endeavors, focused on the life sciences on the one side and bioengineering on the other, must be pushed forward in order to fully utilize both DoD-critical products. While all of the biomarkers may be used in a clinical setting as ELISA tests, they are of little use to fielded troops. Conversely, a DBBM is of little value without validated DoD-relevant biomarker assays permitting the immediate analyses of any number of conditions for which appropriate real-time intervention may play a significant role in not only determining immediate warfighter decrement, but also protecting the future long-term health of that warfigher.

Figure 4: Conceptual image of fieldable DBBM unit

2. JOURNAL-BASED BIOMARKER PRE-VALIDATION

2.1 Baseline Standardization

2.1.1 Baseline Standardization Summary

Creatinine concentrations in urine using D-serine dosages constituting low level toxicity in the rat demonstrated significant variation between controls and dosed groups during time period 0-96 hrs post-dose, with all test groups returning to an equivalent baseline after 96 hrs. Therefore, in this specific nephrotoxin model (D-serine), normalizing biomarker concentrations to creatinine levels at time points prior to 96 hrs may not be valid. We propose the use of a 'baseline-adjusted creatinine concentration' (BACC) value to normalize biomarker concentrations which will allow study-to-study comparisons while adjusting for urine and creatinine fluctuations found in kidney injury biomarker discovery models.

2.1.2 Baseline Standardization Introduction

In order to present urinary biomarker concentration data, some manner of standardization may be used to adjust for variation in urine volumes. For blood/serum based biomarker assays, the average blood volume of an individual will remain relatively constant under normal conditions and constant body weight (Guyton et al., 2000). Therefore blood volume provides little variability for biomarker determinations. However, for urinary biomarkers, total urine volume variability does pose problems in reporting biomarker values capable of lateral comparison between animals, studies, and models. In publications for urinary AKI biomarkers, these data may be adjusted using a ratio of biomarker to creatinine concentration (Zhou et al., 2008). However, creatinine levels are known to vary with kidney injury impairing glomerular filtration rate (Kampmann et al., 1981). Indeed, total creatinine has been used as a clinical test for kidney damage (Bauer et al., 1982; Levey, 1990) and is known to vary with age, muscle mass/metabolism, and hydration (Barr et al., 2005). Thus, the use of total creatinine concentration to normalize urine marker data across studies and test models may not be valid. Since changes in urinary volume will significantly affect concentration of analytes in urine samples, it is essential to normalize urinary analyte measurements to compensate for the fluctuation in the urinary volume that may or may not be associated with compromised renal functions. Since creatinine is usually produced at a fairly constant rate in the body (with the previously mentioned caveats), use of urinary creatinine concentration for normalization in most biomarker discovery research is reasonable. However, this method may not be suitable for normalizing urinary biomarkers for renal injuries since urinary creatinine concentration is significantly affected by renal dysfunctions. As models of renal injuries (diseased- or nephrotoxin-induced) may result in a decreased glomerular filtration rate (GFR), this decrease in creatinine clearance will lead to a concomitant decrease in urinary creatinine concentration. Consequently, this normalization method will likely produce overestimation of biomarker concentrations, and the values cannot be easily compared from study to study.

Some reports eliminate this urinary volume variable by use of urinary excretion rate (UER) calculations (Rigas et al., 2001) in which the reported biomarker data is determined by calculating biomarker concentration in urine multiplied by urine void volume divided by bladder

accumulation time. The data is therefore reported as mass, avoiding urine volume variability. However, this method does not work well in animal and some human studies, where the bladder must be completely voided and time assessed for each collection to complete such calculations. For 'spot' (untimed small volume urine collection) testing, generally the desired mode of biomonitoring, these values will not be available. Current guidelines published by the World Health Organization (WHO) use creatinine concentrations to validate a 'spot' sample assay for environmental toxin monitoring in urine, and recommends testing to be considered valid only in case where the range creatinine is found to be 30 to 300 mg/dL to adjust for urine dilutions (Lauwerys et al., 1993).

We sought to examine creatinine levels and urine volumes at collection time points in our D-serine rat nephrotoxin model to see if creatinine levels have minimal variation throughout the study. If the creatinine concentrations do not provide an adequate method for adjusting for variability in urine volume (creatinine-adjusted), we also examined the use of measured urine volumes for normalization of biomarker data (urine volume-weighted values). Such attention to marker data evaluation is important in pre-validation and validation studies and for comparing marker results to outside published data for accuracy and repeatability as a surrogate endpoint for kidney damage (Coca et al., 2008).

2.1.3 Creatinine Quantitation, Assumptions, and Procedures

Urine and Serum Collection

More detailed descriptions of the animal studies are described in *Biomarkers of Exposure to Toxic Substances, Vol 1* (DelRaso et al., Sep 2009). However, for clarity of this section, we include brief descriptions of each study.

D-serine Study 1

Male Fischer 344 rats were dosed with 5, 20, 50, 200 or 500 mg/kg D-serine in saline, whereas control animals were dosed with saline alone. Five animals were included in each dosing group with the exception of the 200 mg/kg group in which only four animals were included. Urine was collected in metabolic cages from animals prior to dosing and at 24, 48, 72 and 96 hours post-dosing. One percent sodium azide, a bacteriostatic preservative, was added to each sample, and urine was frozen at -20°C within 1 hour of end point collection.

D-serine Study 2U (urine)

Male Fischer 344 rats were dosed with 200 or 500 mg/kg D-serine in saline, whereas control animals were dosed with saline alone. Five animals were included in each dosing group with the exception of the control group in which only four animals were included. Urine was collected from animals prior to dosing and at 12-hour intervals up to 168 hours post-dosing. Protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) equal to 1/100 of the urine volume was added, and urine was frozen immediately at -20°C before being moved into a -80°C freezer within 1-7 days.

D-serine Study 2S (serum)

Male Fischer 344 rats were dosed with 200 or 500 m g/kg D-serine in saline, whereas control animals were dosed with saline alone. Ten animals were included in each dosing group. Five of the ten animals were sacrificed 12 hours post-dosing. The remaining five animals were sacrificed 24 hours post-dosing. Blood was collected from the inferior vena cava.

Creatinine ELISA Protocol

Creatinine assays were performed as described in the R&D Systems, Inc. Parameter™ Creatinine Assay kit instructions. First, urine samples were diluted 1:20 with 18 mΩ water. Fifty microliters of diluted sample were added to the wells of 96-well microplates. One hundred microliters of alkaline picrate solution were added to each well and incubated for 30 minutes at room temperature. The absorbance (optical density [OD]) was measured at 490 nm with a microplate reader (Molecular Devices SpectraMax M2^e).

2.1.4 Creatinine Concentration and Urine Volume Results

Creatinine concentrations were plotted as mg/dL versus post-dose time collection point, a standard method for presenting biomarker profiles. Creatinine evaluation of urine samples collected every 24 hrs (Study 1) demonstrate a significant decrease in all sets at 3 hrs (Figure 5). For control animals, creatinine returned to pre-dose levels after 96 hrs, whereas at 96 hrs both the 200 and 500 mg/kg dose groups remained at less than half pre-dose levels. A closer examination was conducted of the creatinine profile using 12 hr collection points spanning a total of 168 hrs (Figure 6). This additional data duplicate creatinine values from Study 1, and further indicate that creatinine levels in both dose groups start a gradual return at 72 hrs to pre-dose levels in a dose-dependent manner (Table 1).
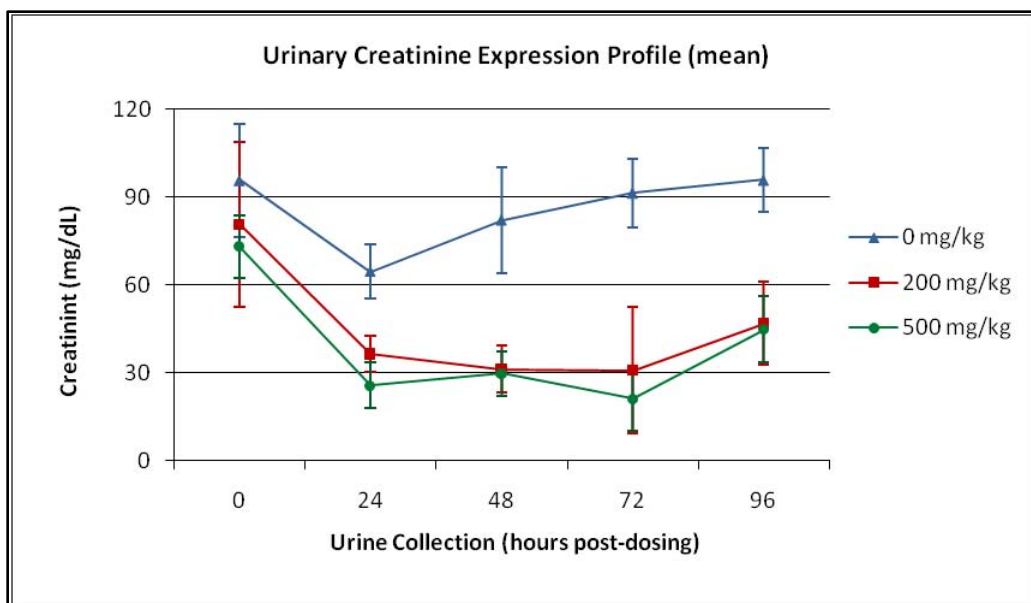
Figure 5: Measurement of urinary creatinine versus time after D-serine administration (Study 1)
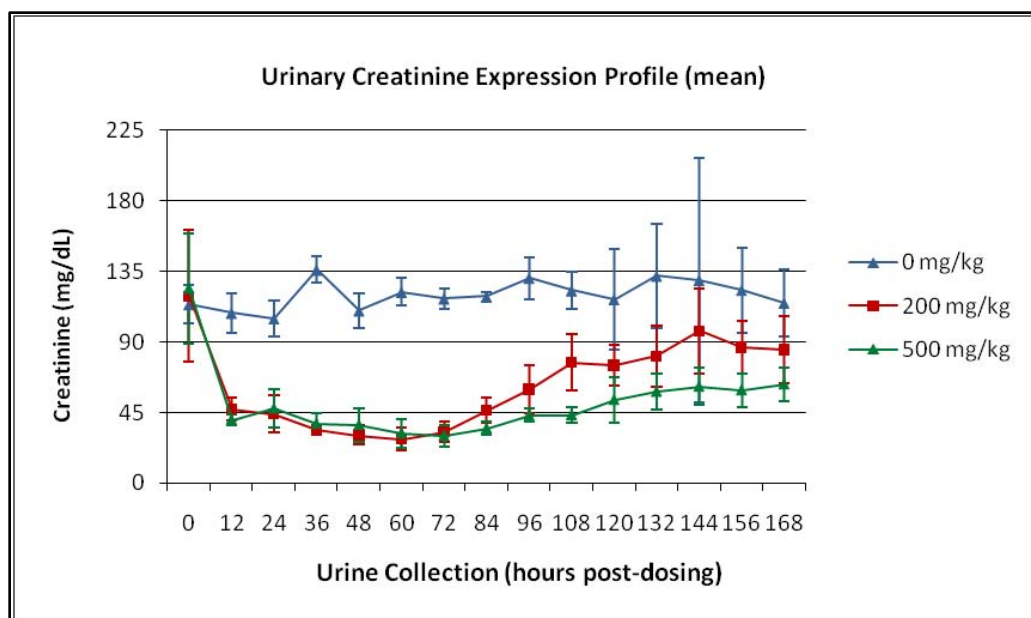
Figure 6: Measurement of urinary creatinine versus urine collection time after D-serine administration (Study 2U)

Table 1: **Significance of creatinine differences between control and dose groups at various collection times using creatinine (mg) urine volume-adjusted**

Statistical analysis using one-way ANOVA followed by multiple comparisons if the ANOVA was significant (p>0.05).

Study 2U		
Time	**Conclusion**	Remarks
0 hours	No significant difference among the 3 groups	
12 hours	Group 200 higher than Group 500, no other pairs differ significantly	
24 hours	Group 0 higher than Group 500, no other pairs differ significantly	
36 hours	**Group 0 higher than both Group 200 and Group 500, Groups 200 and 500 not significantly different**	
48 hours	**Group 0 higher than both Group 200 and Group 500, Groups 200 and 500 not significantly different**	
60 hours	**Group 0 higher than both Group 200 and Group 500, Groups 200 and 500 not significantly different**	
72 hours	Group 500 lower than Groups 0 and 200, Groups 0 and 200 not significantly different	
84 hours	Group 200 higher than Group 500 and 0, Groups 0 and 500 do not differ significantly	Failed assumption of equal variance; used weighted least squares as a remedy
96 hours	**No significant difference among the 3 groups**	

2.1.5 Discussion of Normalized Creatinine Concentrations

Urinary creatinine excretion (urine volume x creatinine concentration) in healthy subjects is considered to be relatively constant, urinary metabolite levels are frequently reported in creatinine-adjusted values. Creatinine-adjusted concentrations of biomarkers, therefore, supposedly correct for differences in urine volumes between subjects/animals. While studies concluded that creatinine is an adequate substitute for urine volume in laboratory animals and humans (Klante et al., 1997), more recent studies have challenged that notion. Additional studies (Fortin et al., 2008) found intra-individual variation in creatinine excretion rates as high as 6.6-fold over a 24 hour period suggesting that creatinine-adjusted concentrations of a metabolite biomarker measured in two separate urine samples obtained the same day from one subject could differ substantially (Dia et al., 2008). This study also concluded that the units that are chosen to

present biomarker data may invalidate comparisons between samples and introduce bias in either urine flow weighted or creatinine-adjusted values.

To examine our data either as creatinine-adjusted or urine volume-weighted values, we first examined urine output in the three groups (control, 200 mg D-serine/kg, 500 mg D-serine/kg) per time point collected. Since urine volume data were not collected in Study 1, this analysis was conducted on Study 2U data only. Examination of urine output versus time (Figure 7) indicates several interesting points. First, urine volume dramatically increases in both dose groups compared to control. Additionally, as seen in several biomarker discovery studies (Bottini 2002; Hoorn et al., 2005), diurnal effects on urine output are clearly seen (Figure 7, 0 mg/kg group). As the rats are naturally more active in nocturnal hours, a marked increase in urine output is seen in control animals during every dark cycle. Urine volumes for all groups, after variation 12-108 hrs, move towards a common baseline value at 132 hrs (51/2 days).
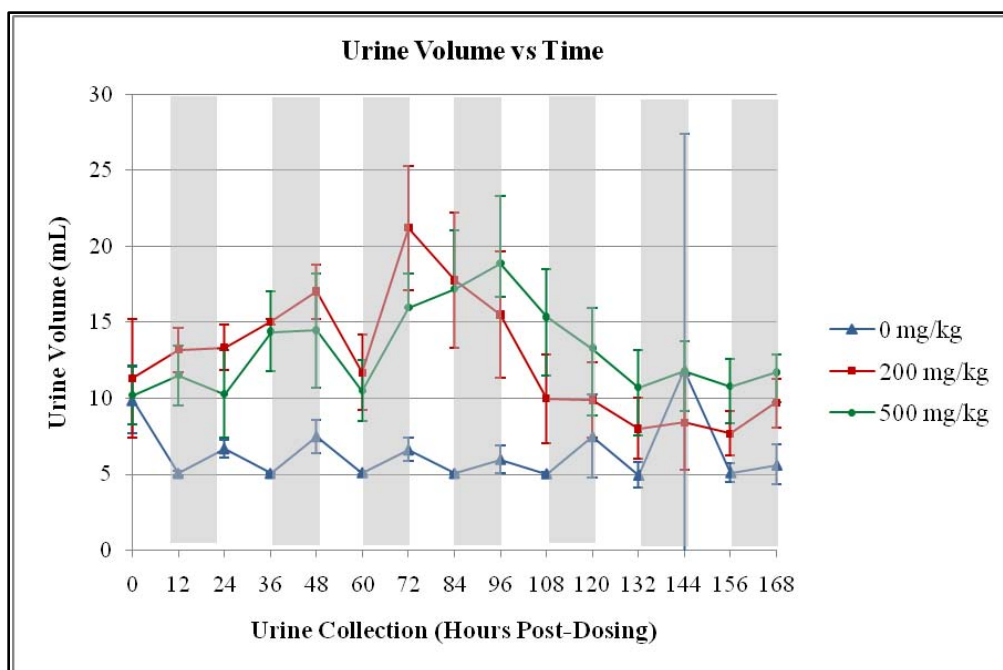
Figure 7: **Measurement of total urinary output at sample collection time points in control and dosed animals (Study 2U)**
Light/dark cycles of animal room are indicated.

Adjusting values using a urine volume-weighted approach (Figure 8) demonstrated that creatinine levels still exhibited significant variability between time points 12 to 96 hrs (arrows).
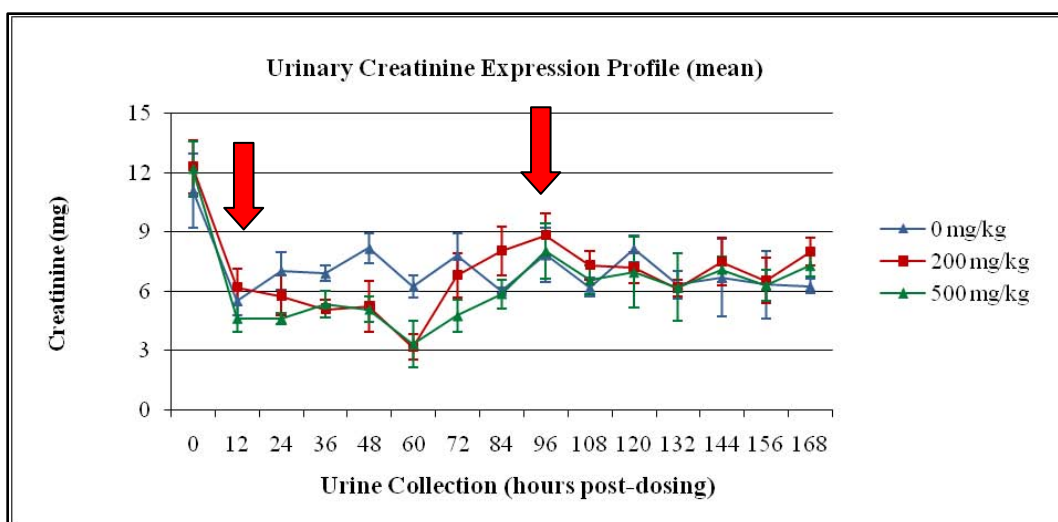
Figure 8: **Measurement of urine volume-weighted creatinine values versus time point after D-serine administration (Study 2U)**

One method that may be used to avoid these problems would be the use of urine volume-weighted creatinine baseline values which we will term 'baseline-adjusted,' or Baseline Adjusted Creatinine Concentration (BACC):

$$\text{BACC} = \text{Measured creatinine concentration} \times \frac{\text{Baseline creatinine excretion (mg)}}{\text{Postdose creatinine excretion (mg)}}$$

In theory, this should address both the variation in urinary volume and the change in creatinine excretion. As seen both creatinine-adjusted or urine value calculations may introduce bias into reported values without careful examination of both values in control groups. None of the methods examined [Creatinine (mg/dL), total creatinine (mg) urine volume-weighted] provide a relatively stable baseline value throughout the entire study on which to normalize biomarker concentrations and provide a value that may be quantitatively used in comparisons to other studies. One may argue, at least in the D-serine study conducted, total creatinine (mg) may be reasonably used as a baseline value after 96 hrs (Figure 8). However, we propose the use of BACC for reporting urine kidney biomarker data or for urine biomarker discovery in models that may have kidney injury as a primary or secondary effect.

It is unclear why total creatinine (mg) levels were high in all three groups at zero time point (pre-dose). Pre-dose urine samples were collected prior to injections therefore should not reflect stress induced response due to handling/injections. As it is seen in all three groups prior to injection, it is not associated with any nephrotoxic event but due to some undefined confounding factor common to all three groups. Individual animals were placed at random at different cage levels to avoid biomarker variation due to cage stacking. One possible source of this variation

may be the placement in the metabolic cages only 24 hrs prior to injection. Animals had been placed in the metabolic cages for 1 hr each day prior to being placed full time one day before injection. It is thought that the additional daily handling of the animals and new environment may have induced this high creatinine value. The 'normal' creatinine baseline value seen during this study is 6.66 mg (calculated from time points 108 to 168 hrs, $\sigma = 1.25$, % CV = 18.74), and this value is not reflected in the pre-dose samples. The normal range of creatinine in rat urine is approximately 6 mg (Van Pilsum 1963). An examination of urine creatinine levels at least three days prior to injection would have given better pre-dose baseline information and provide clarification on these values. In addition, we anticipate that full time placement in the metabolic cages a minimum of three days prior to dosing would have given creatinine values in baseline range. Another source of these high values was the incorrect collection of the pre-dose urine which reflected a 24 hr collection pre-dose instead of a 12 hr pre-dose collection.

If one examines the study data using BACC values instead of creatinine-adjusted or urine volume-weighted (shown here for osteopontin data), one can obtain a biomarker profile capable of direct comparison to similarly graphed data from other studies (Figure 9). In this study data, the abnormally high creatinine value at pre-dose, along with errors discussed above, do not allow a good interpretation of the value of such normalization. To examine how such data may graph if the creatinine pre-dose values had been generated, we also graphed the BACC normalization of osteopontin data assuming a pre-dose creatinine value of 6.5 mg (Figure 10).
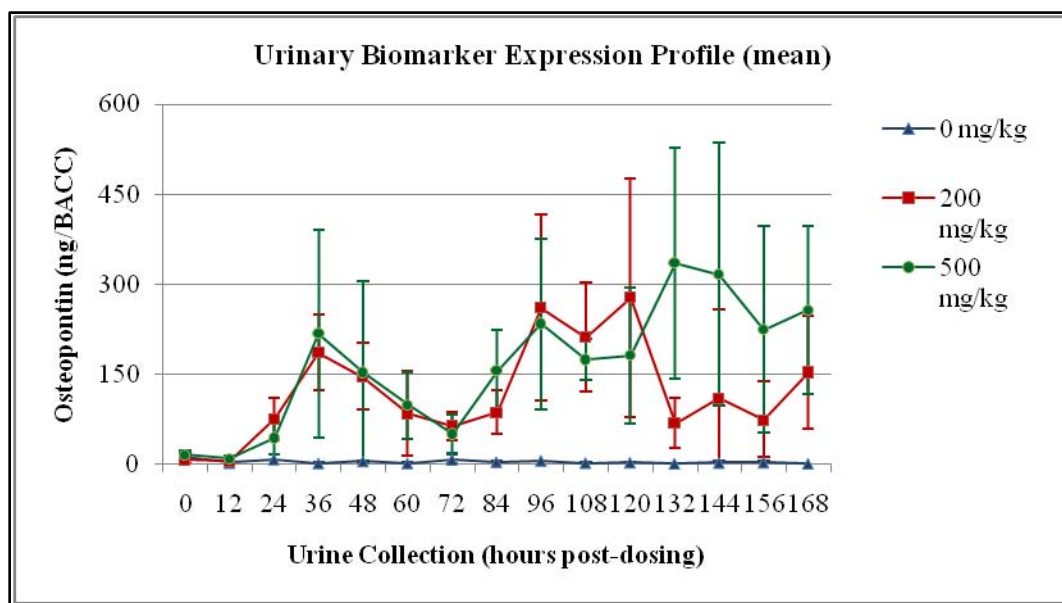
Figure 9: **Biomarker profile using osteopontin study data normalized to BACC values**
BACC values calculated on study creatinine baseline and post-dose urine volume-weighted values.
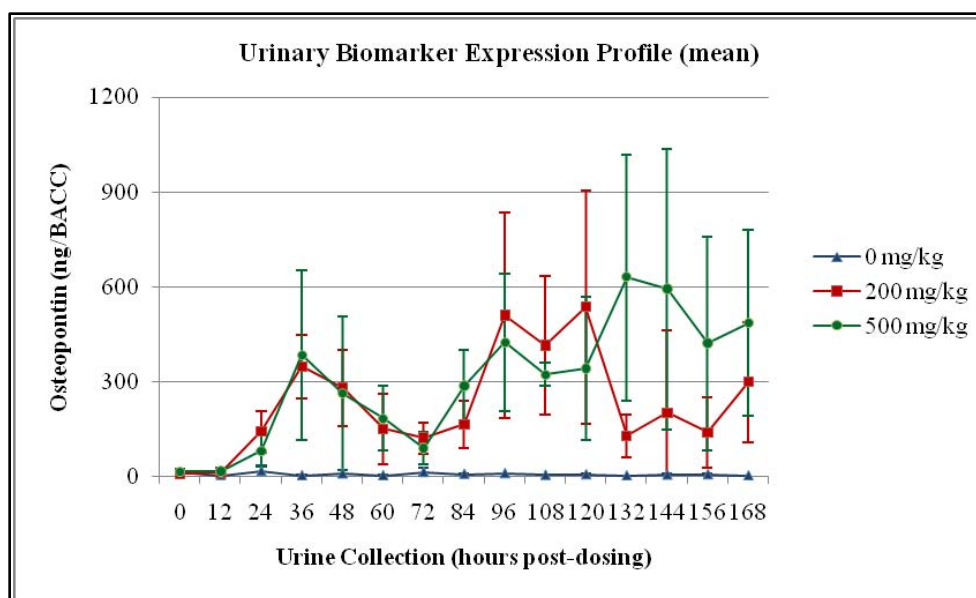
Figure 10: Biomarker profile using osteopontin study data normalized to BACC values using 6.5 mg
BACC values based on published 'normal' urine creatinine urine volume-weighted value of 6.5 mg and study post-dose urine volume-weighted values.

While the profile pattern is replicated, the ng/BACC values are increased 1.6, 1.9, and 1.8 fold (for dose groups 0, 200, and 500 mg/kg, respectively) in Figure 10, and probably reflect numbers that would more closely reflect normalization calculations with the previously discussed study changes in animal handling and pre-dose collections.

For biomonitoring, a semi-quantitative change in specific biomarker levels are generally used as an indication of disease or injury state, versus the more quantitative values generated within the clinical laboratory, and such difficulties are relatively mute if addressed during pre-validation and validation research. However, for these pre-validation studies examining biomarker response in multiple nephrotoxin models (both RHPB and published), care must be taken to acknowledge and address these concerns. For the purpose of data presentation within this report, creatinine-adjusted and urine volume-weighted, and BACC calculations (using 6.5 mg pre-dose value) will be presented. Additional data on the pre-dose baseline creatinine concentrations would be needed to fully validate our suggested animal study changes and therefore the BACC mode of calculations, but the BACC graphed data will be included for future examinations.

2.2 Metalloprotease inhibitor 1 (TIMP1)

2.2.1 TIMP1 Summary

The metalloproteinase inhibitor TIMP1 has been implicated as a potential biomarker predictive of kidney injury in several published studies and was therefore included in this study for its utility as an indicator of low level kidney damage. The protein expression profile of TIMP1 in urine was examined in a time -dose response study using the known nephrotoxin D-serine. Male Fischer 344 rats were dosed with 0, 5, 20, 50, 200, or 500 mg/kg D-serine in saline. Urine samples were kept cold during collection periods of 24, 48, 72 and 96 hours post-dose and frozen within 1 hour at each time point. After the completion of the study, the samples were assayed for TIMP1 by ELISA. Biomarker levels remained constant for control animals and in animals dosed with 5, 20, and 50 mg/kg D-serine throughout the time course. Animals dosed with 200 and 500 mg/kg D-serine demonstrated a significant increase at 24 hours post-dose, with levels dropping to pre-dosing levels at 72 hours post-dose, followed by a smaller but significant secondary increase at 96 hours post-dose. A second time-course experiment was conducted in order to confirm the biphasic nature of the peak response times of the biomarker. In the second study, urine samples were collected as before with the exception that the samples were taken at 12 hour intervals, collected for a longer 168 hour period, and protease-inhibitor added to the samples prior to freezing. The TIMP1 biomarker was quantitated as before. As previously observed, significant increases in TIMP1 were observed at 24 hours post-dose. Levels again dropped near baseline levels at 48 hours post-dose, with a slight increase at 96 to 120 hours post-dose. However, these levels did not reach the concentrations as previously observed. Serum samples were also assayed by ELISA for TIMP1 using blood collected at 12 and 24 hours post-dose in the 0, 200 and 500 mg/kg D-serine animals. No statistically significant differences in TIMP1 levels were observed between the 12 and 24 hour time points of all three groups. A one-way ANOVA between dosing groups on pooled 12 and 24 hour data showed no significant differences in the expression of TIMP1 between the control and either dosing group or between the 200 and 500 mg/kg dosing groups. Along with the existing journal based data, these expression response profiles to a nephrotoxin indicate that urinary TIMP1 detection is a potential noninvasive strategy to predict early onset of kidney damage via toxins within 24 hrs of acute exposure. TIMP1 changes in the blood concomitant with kidney damage were observed, but the changes were too small to be significant in this model, in conflict with previously published data (Hörstrup et al., 2002).

2.2.2 TIMP1 Introduction

Extracellular matrix (ECM) degradation enzyme families consist of zinc-dependent matrix metalloproteinases (MMPs) and their respective inhibitors (TIMPs) which play a role in the maintenance of connective tissue homeostasis (Chen, 1992). Changes in expression levels of MMPs or TIMPs can lead to changes in degradation, and is thought to allow tumor cells to invade other regions (Polette et al., 2004). TIMP1 increases seem to also lead to other disease states (Sato et al., 1992; Bhuvarahamurthy et al., 2006). A subset of MMPs consisting of MMP1, MMP3, and latent MMP2 and MMP9 are regulated by TIMP1 and TIMP2. It has been seen that TIMP1 binds to MMP9 preferentially over other MMP forms (DeClerck et al., 1991).

TIMP1, Tissue inhibitor of metalloproteinase-1, (GeneID: 7076), was identified as a potential biomarker for this study using literature searches of published biomarker data. Rat TIMP1 is a 28-35 Kdal glycoprotein and, while produced in a wide range of cell types, is primarily expressed in the distal tubule, collecting ducts, and thick ascending limb of Henle in the kidney. TIMP1 has also been detected in oxygen-depleted proximal tubular epithelial cells.

An increase in the synthesis and degradation of collagenous and non-collagenous matrix proteins are seen in chronic kidney disease (CKD), and these increases were also reflected in higher levels of collagen fragments in the serum and urine of CKD patients (Soylemezoglu et al., 1997). Additional studies examining serum and urine for other markers indicative of kidney disease indicated that TIMP1 and tenascin increased in CKD patients. In this study, TIMP1 was shown to be at a lower level in urine compared to serum (6 fold verses 18 fold increase), but both increases were thought to be related to levels of renal fibrosis (Hörstrup et al., 2002). TIMP1gene expression was also examined in renal cell carcinoma and identified as a potential biomarker using RNA expression and immunostaining in renal cell carcinomas (Bhuvarahamurthy et al., 2006) and in anti-neutrophil cytoplasmic antibody (ANCA)-associated glomerulonephritis (Sanders et al., 2004). More recent data using gene expression changes using a chronic nephrtotoxin exposure model (either Bacitracin, Gentamicin, Cisplatin, Vancomycin) in the rat indicated that TIMP1 expression levels increased up to 30 fold after five days using Bacitracin (380 mg/kg/d, daily dose), with much smaller fold increases with other tested nephrotoxins (Wang et al., 2008). Such studies provide data to indicate that the TIMP1 protein may work as a kidney injury biomarker in both urine and serum, with significant changes seen in both acute and chronic kidney disease as well as nephrotoxin models.

2.2.3 TIMP1 Methods, Assumptions, and Procedures

Urine and Serum Collection

More detailed descriptions of the animal studies are described in *Biomarkers of Exposure to Toxic Substances, Vol 1* (DelRaso et al., Sep 2009). However, for clarity of this section, we include brief descriptions of each study. Studies were conducted under a program of animal care accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International and in accordance with the "Guide for the Care and Use of Laboratory Animals," National Research Council (1996). Studies were conducted under approved Air Force Research Laboratory Institutional Animal Care and Use Committee Protocol F-WA-2003-0074-A.

D-serine Study 1

Male Fischer 344 rats were dosed with 5, 20, 50, 200 or 500 mg/kg D-serine in saline, whereas control animals were dosed with saline alone. Five animals were included in each dosing group with the exception of the 200 mg/kg group in which only four animals were included. Urine was collected in metabolic cages from animals prior to dosing and at 24, 48, 72 and 96 hours post-dose. One percent sodium azide, a bacteriostatic preservative, was added to each sample, and urine was frozen at -20°C within 1 hour of end point collection.

D-serine Study 2U

Male Fischer 344 rats were dosed with 200 or 500 mg/kg D-serine in saline, whereas control animals were dosed with saline alone. Five animals were included in each dosing group with the exception of the control group in which only four animals were included. Urine was collected from animals prior to dosing and at 12-hour intervals up to 168 hours post-dose. Protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) equal to 1/100 of the urine volume was added, and urine was frozen immediately at -20°C before being moved into a -80°C freezer within 1-7 days.

D-serine Study 2S

Male Fischer 344 rats were dosed with 200 or 500 mg/kg D-serine in saline, whereas control animals were dosed with saline alone. Ten animals were included in each dosing group. Five of the ten animals were sacrificed 12 hours post-dose. The remaining five animals were sacrificed 24 hours post-dose. Blood was collected from the inferior vena cava.

Validation of RayBio® TIMP1 ELISA Kit for Use in a Urine Matrix.

ELISA Kit Evaluation 1

Enzyme-linked immunosorbent assays were performed using RayBio® rat TIMP1 ELISA kits (RayBiotech, Inc., GA). For four urine samples expected to have measurable levels of TIMP1, 3-point, 4-fold serial dilutions were performed, beginning at 1:2 and ending at 1:32. Reference standard material at concentrations of 0, 50, 500 and 5000 pg/mL of TIMP1 were spiked into 1:2, 1:8, and 1:32 dilutions of a baseline urine sample.

ELISA Kit Evaluation 2

A 3-point, 2-fold serial dilution was performed, beginning at 1:32 and ending at 1:128. Reference standard material at concentrations of 0, 50 and 5000 pg/mL of TIMP1 were spiked into a baseline urine sample diluted 1:64. In addition, 0 and 500 pg/mL of TIMP1 were spiked into a baseline urine sample diluted 1:128.

ELISA Kit Evaluation 3

Three samples were diluted 1:16 and 1:32. In addition, TIMP1 was spiked into a baseline urine sample at 0, 50, 500 and 5000 pg/mL. The urine was diluted 1:16.

Urine and Serum Analysis by ELISA

Enzyme-linked immunosorbent assays were performed as described in the RayBio® rat TIMP1 ELISA kit protocol. First, urine samples were diluted 1:32 with assay diluent A (30 mL of animal serum with 0.09% sodium azide). Serum samples were diluted 1:4 with assay diluents A. One hundred microliters of diluted sample were added to the wells of 96-well microtiter plates coated with anti-rat TIMP1 and incubated overnight at 4°C. Plates were washed 4 times

with wash buffer. One hundred microliters of biotinylated anti-rat TIMP1 antibody diluted in assay diluent B were added and incubated for 1 hour at room temperature. Wells were washed four times, 100 uL of streptavidin solution diluted in diluent A was added to the wells, and plates were incubated for 45 minutes at room temperature. Wells were washed five times, 100 uL of TMB one-step substrate reagent (3,3',5,5'-tetramethylbenzidine in buffered solution) added, and plates incubated 30 minutes at room temperature in the dark. Reactions were stopped by the addition of 50 uL of 2 M sulfuric acid. The absorbance (optical density [OD]) was measured at 450 nm with a microplate reader (Molecular Devices SpectraMax M2[e]).

Statistical Analysis

All data were the average of duplicate determinations. The results were expressed as mean values ± standard deviations (SD). Statistical methodology included analysis of variance (H0: $\mu_1 = \mu_2 = \ldots = \mu_k$ versus H_a: $\mu_i = \mu_j$). When a significant difference was found at $\alpha = .05$, Tukey's multiple comparisons were performed to identify which dosing groups differed significantly. To ensure validity of the analysis of variance, the Kolmogorov-Smirnov test of normality and Bartlett's test for equal variances were performed on the residuals. Where normality was not justified, the Kruskal-Wallis test was performed in lieu of the analysis of variance, followed by Tukey's multiple comparisons as appropriate. When the assumption of equal variances failed, transformations were performed to stabilize the variance.

2.2.4 TIMP1 Results and Discussions

TIMP1 ELISA Kit Evaluations

The RayBio[®] rat TIMP1 ELISA kit is validated for use with rat serum and plasma but not urine. Therefore, the kit was evaluated by three independent analyses in order to assess the kit's capability of detecting TIMP1 in rat urine. First, the recovery efficiency of TIMP1 from the urine matrix was assessed through a spike and recovery experiment. Second, the kit's capability of detecting TIMP1 from in-house rat urine samples expected to have measurable levels of the protein was assessed. Third, the linearity of the results obtain from multiple dilutions of a urine sample was assessed.

ELISA Kit Evaluation 1

The kit insert did not recommend a starting dilution of samples. Therefore, the first evaluation was designed to assay urine samples expected to have relatively high levels of TIMP1 at multiple dilutions to establish the appropriate sample dilution. Additionally, a spike and recovery experiment was performed to assess the recovery efficiency of TIMP1 from the urine matrix. Various dilutions of a baseline urine sample were prepared in assay diluent. The urine dilutions covered the range of dilutions anticipated to be used in regular sample testing. The standard material was then spiked into the urine dilutions and recovery was assessed. For three out of the four urine samples, the ODs were above the range of the standard (Figure 11) at the 1:2 dilution, and for two out of the four urine samples, the ODs were above the range of the standard at the 1:8 dilution. All samples had ODs within the range of the standards at a dilution of 1:32. While dilutional linearity for points 1:8 and 1:32 was demonstrated for sample 4 with an

intra-assay CV of only 13.8%, dilutional linearity was not demonstrated for sample 2 with an intra-assay CV of 61.3% for points 1:2 through 1:32 (Table 2). Recoveries of 50, 500 and 5000 pg/mL of TIMP1 from urine diluted 1:2 were 31.2, 1.0 and 8.4%, respectively. Recoveries of the protein from urine diluted 1:8 were 147.6, 54.1 and 35.0%, respectively. Recoveries at the 1:32 dilution were 111.0, 104.7 and 64.3%, respectively (Table 3). Dilutional linearity was not observed with intra-assay %CV of the various spikes ranging from 61.6 to 97.4% (Table 4).

The ODs of samples expected to have relatively high levels of TIMP1 indicated that this protein can be detected in urine. However, both the dilution experiment and the spike and recovery experiment indicated that samples cannot be diluted at low as 1:2 or even 1:8. A more consistent recovery of TIMP1 was observed at a urine dilution of 1:32. A second dilution experiment was performed to include a two-fold serial dilution beginning at 1:32 and ending at 1:128 of a urine sample expected to contain measurable levels of TIMP1. In addition, a spike and recovery experiment in which 0, 50 and 5000 pg/mL were spiked into urine diluted 1:64 and 1:128 was designed.

TIMP-1 Standard Curve

4-P Fit: y = (A - D)/(1 + (x/C)^B) + D:	A	B	C	D	R^2
● Std (Standards@Exp01: Conc vs MeanValue)	0.077	1.158	1994.502	2.768	1

Curve Fit Option - Fixed Weight Value: 1
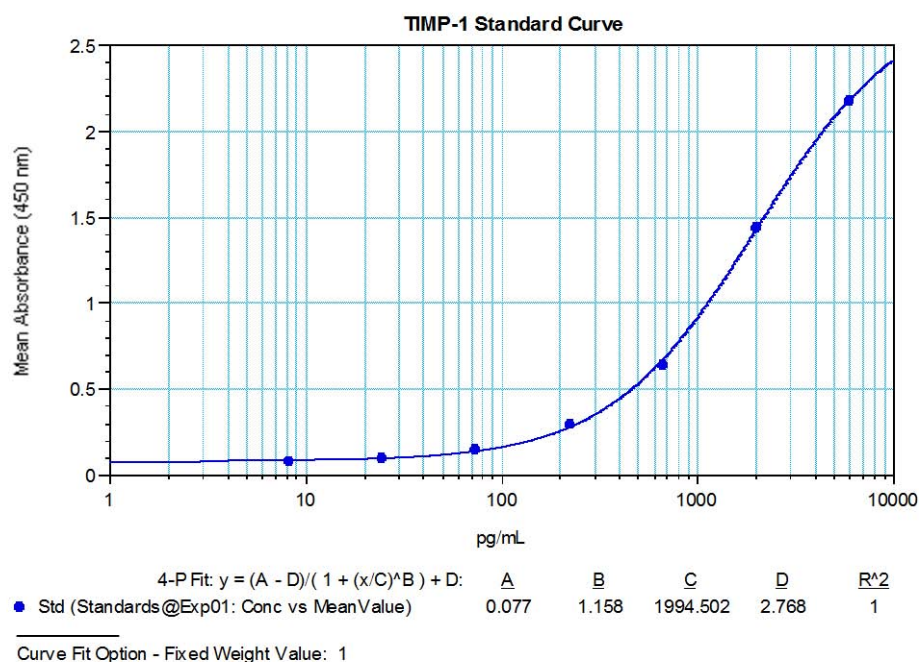
Figure 11: TIMP1 Standard Curve

Table 2: Analysis of test animal urine at dilution factors of 2, 8 and 32
Outside of the range of the standard.

Sample	Dilution Factor	Mean OD	Result (pg/mL)	Duplicate %CV	Adjusted Result (µg/mL)	Mean	%CV
1	2	*2.389	NA	NA	NA	NA	NA
	8	*2.322	NA	NA	NA		
	32	1.814	3639.6	7.2	116466.2		
2	2	1.714	3154.6	4.1	6309.2	16461.0	61.3
	8	1.395	2074.9	1.4	16598.86		
	32	0.727	827.3	5.7	26474.9		
3	2	*2.575	NA	NA	NA	NA	NA
	8	*2.175	NA	NA	NA		
	32	1.327	1903.4	0.4	60907.3		
4	2	*2.331	NA	NA	NA	34047.4	13.8
	8	1.849	38.40.6	10.4	30724.7		
	32	0.955	1167.8	11.7	37370.0		

Table 3: Spike and recovery of 0, 50, 500 and 5000 pg/mL TIMP1
*TIMP1 (reference standard material, RSM) aliquoted into 1:2, 1:8, and 1:32 dilutions of baseline urine sample; * Outside of the range of the standard.*

Spiked concentration (pg/mL)	Mean OD	Mean Result (pg/mL)	Replicate CV%	Adjusted Result (spike-0 ng/mL)	Urine Dilution	Percent Recovery
0	0.949	1157.1	5.8	NA	1:2	NA
50	0.959	1172.7	0.0	15.6	1:2	31.2
500	0.952	1161.9	6.3	4.8	1:2	1.0
5000	1.180	1575.6	3.3	418.5	1:2	8.4
0	0.726	826.5	2.5	NA	1:8	NA
50	0.780	900.3	3.7	73.8	1:8	147.6
500	0.912	1097.2	3.9	270.7	1:8	54.1
5000	1.563	2577.4	3.9	1750.9	1:8	35.0
0	0.293	309.6	3.3	NA	1:32	NA
50	0.344	365.1	11.1	55.5	1:32	111.0
500	0.731	833.1	0.4	523.5	1:32	104.7
5000	1.793	3523.8	2.8	3214.2	1:32	64.3

Table 4: **Dilutional linearity TIMP1 first dilution evaluation; Urine samples diluted 1:2, 1:8 and 1:32**.

Spiked concentration (pg/mL)	Mean Adjusted Result (pg/mL)	Linearity of urine dilution (%CV)
50	48.3	61.6
500	266.3	97.4
5000	1794.5	77.9

ELISA Kit Evaluation 2

The dilutional linearity for points 1:32 to 1:128 was demonstrated, with an intra-assay CV of 12.7% as shown in Table 5. The recoveries of TIMP1 from urine diluted 1:64 were low, ranging from 0-54.6%. Recovery of the protein from urine diluted at 1:128 was 115.4%. However, recovery at the 1:128 dilution was based on a single spike (Table 6). Based on evaluations 1 and 2, the lowest recommended starting dilution of urine to be assayed with the RayBio® rat TIMP1 ELISA kit is 1:32. Recoveries of TIMP1 from spiked urine ranged from 54-115% in the acceptable dilution levels. A third dilution experiment was performed in order to assess can be diluted 1:16, rather than 1:32, without encountering matrix interference. Three samples were diluted 1:16 and 1:32 in order to assess dilutional linearity. In addition, a spike and recovery experiment was performed. TIMP1 was spiked into a baseline urine sample at 0, 50, 500 and 5000 pg/mL. The urine was diluted 1:16.

Table 5: **Dilutional linearity TIMP1 second dilution evaluation: Urine samples diluted 1:32, 1:64 and 1:128.**

Sample	Dilution Factor	Mean OD	Result (pg/mL)	Duplicate %CV	Adjusted Result (μg/mL)	Mean	%CV
1	32	1.724	4201.	7.6	134437.7		
	64	1.198	2217.	3.3	141909.3	148879.0	12.7
	128	0.835	1330.	6.6	170289.9		

Table 6: **Spike and recovery of 0, 50, 500 and 5000 pg/mL TIMP1**
TIMP1 (RSM) aliquoted into 1:64 and 1:128 dilutions of baseline urine sample.

Spiked concentration (pg/mL)	Mean OD	Mean Result (pg/mL)	Replicate CV%	Adjusted Result (spike-0 ng/mL)	Urine Dilution	Percent Recovery
0	1.164	2123.0	4.8	NA	1:64	NA
50	1.037	1789.7	2.9	-333.3	1:64	NA
5000	1.846	4854.5	5.0	2731.5	1:64	54.6
0	0.698	1057.5	10.8	NA	1:128	NA
500	0.972	1634.4	0.6	576.9	1:128	115.4

ELISA Kit Evaluation 3

The dilutional linearity for points 1:16 to 1:32 is acceptable, with intra-assay CVs ranging from7.4% to 24.4% (Table 7). However, the recoveries of TIMP1 from urine diluted 1:16 were low, ranging from 0-54.3% (Table 8). Based on all three kit evaluations, the lowest recommended starting dilution of urine to be assayed with the RayBio® rat TIMP1 ELISA kit is 1:32.

Table 7: TIMP1 Dilutional linearity between urine samples diluted 1:16 and 1:32.

Sample	Dilution Factor	Mean OD	Result (pg/mL)	Duplicate %CV	Adjusted Result (µg/mL)	Mean	%CV
1	16	1.12	1064.7	4.5	17035.1	18361.7	10.2
	32	0.72	615.3	8.8	19688.4		
2	16	0.58	482.3	11.0	7717.3	9329.8	24.4
	32	0.42	342.0	11.2	10942.2		
3	16	0.77	664.2	25.5	10627.1	11211.9	7.4
	32	0.45	368.7	4.6	11796.8		

Table 8: **Spike and recovery of 0, 50, 500 and 5000 pg/mL TIMP1**
TIMP1 (reference standard material) aliquoted into a 1:16 dilution of baseline urine sample

Spiked concentration (pg/mL)	Mean OD	Mean Result (pg/mL)	Replicate CV%	Adjusted Result (spike-0 ng/mL)	Urine Dilution	Percent Recovery
0	0.608	502.267	6.1	NA	1:16	NA
50	0.608	502.416	7.3	0.15	1:16	0.3
500	0.879	773.873	9.2	271.606	1:16	54.3
5000	1.867	2401.064	2.7	1898.797	1:16	38.0

Examination of TIMP1 in urine from D-serine rat model urine.

D-serine Study 1

The known nephrotoxin D-serine was used to induce sub-clinical kidney damage at the proximal straight tubules in order to evaluate whether TIMP1 could serve as an early predictor of nephrotoxicity. Doses of 20, 50, 200 or 500 mg/kg D-serine were administered intraperitoneally in 0.9% saline. Control animals were dosed with saline alone. Urine samples were collected at 24 hours intervals in a 96 hour period. TIMP1 was quantitated by sandwich ELISA and the biomarker expression profile was determined (Figure 12). Significant biomarker profile changes in TIMP1 were seen in urine only in the 200 and 500 mg/kg D-serine dose groups. No significant profile changes were seen at the 5, 20 or 50 mg/kg D-serine dose groups (data not shown). Kidney histopathology data and clinical chemistry data (TBIL, CREA, BUN, and Total

Protein) confirmed the initiation of kidney damage around the 200 mg/kg dose (data DelRaso et al., Sep 2009). The urine profile of the biomarker peaked at 24 hours, reaching 221,947 pg/mL and 258,820 pg/mL in dosing groups 200 and 500 mg/kg, respectively (Figure 12). At peak expression, dosing groups 200 and 500 mg/kg were significantly larger than the control group. However, the 200 and 500 mg/kg dosing groups were not significantly different from each other ($p<0.05$). At the 72 hour time-point, TIMP1 levels decreased to as low as 28,737 pg/mL and 25,712 pg/mL in dosing groups 200 and 500 mg/kg, respectively. Levels were found to increase again at the 96 hour time point with TIMP1 concentrations reaching 77,687 pg/mL and 82,919 pg/mL in dosing groups 200 and 500 mg/kg, respectively. No plateau was observed in the excretion of TIMP1 during the 96 hour time-course. Instead, TIMP1 may exhibit a biphasic expression profile. It was determined that a longer time-course experiment was needed to clarify any relevancy and extent of the 96 hour peak.
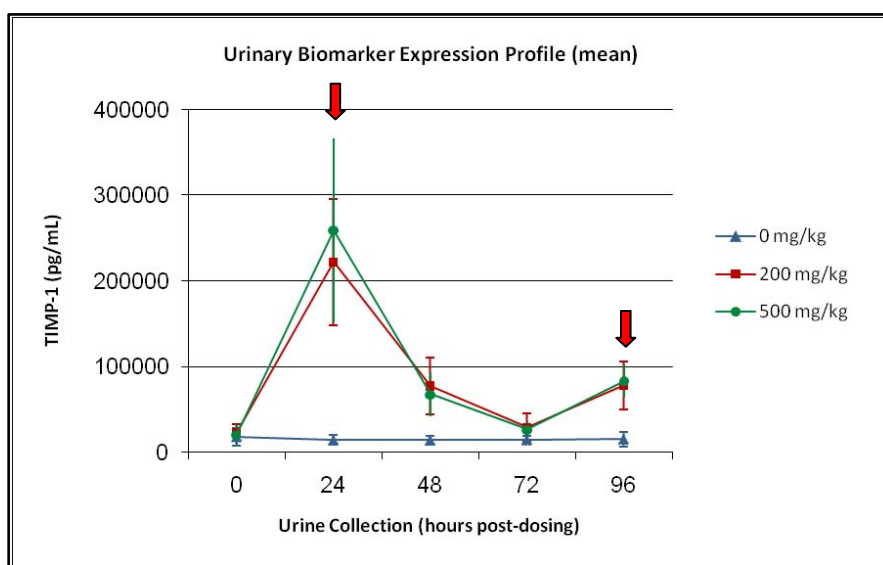
Figure 12: **Measurement of urinary TIMP1 by ELISA over time after D-serine administration (Study 1)**
The biphasic biomarker response is indicated by arrows

D-serine Study 2U

A second time-course experiment was conducted in order to clarify the relevancy of the increase in TIMP1 concentration at the 96 hour time point and to confirm that the TIMP1 marker response is biphasic in nature (Figure 12). Again, D-serine was used to induce sub-clinical kidney damage at the proximal straight tubules. Doses 200 or 500 mg/kg D-serine were administered intraperitoneally in 0.9% saline, with control animals injected with saline alone. Urine samples were collected more frequently and for a longer period than in Study 1, taking samples at 12 hours intervals over a 168 hour period. TIMP1 was quantitated by sandwich ELISA and the biomarker expression profile was determined.

As previously observed in the original time-course experiment, significant biomarker profile changes in TIMP1 were seen in urine in the 200 and 500 mg/kg D-serine dose groups. Kidney histopathology data and clinical chemistry data (TBIL, CREA, BUN, and Total Protein) confirmed that the 200 and 500 mg/kg doses produced sub-clinical kidney damage in this model (data in DelRaso et al., Sep 2009). The urine profile of the biomarker achieved maximal levels at 24 hours, reaching 260,902 pg/mL and 332,615 pg/mL in dosing groups 200 and 500 mg/kg, respectively (Figure 13). At peak expression, dosing groups 200 and 500 mg/kg were significantly larger than the control group. However, the 200 and 500 mg/kg dosing groups were not significantly different from each other ($p<0.05$). At the 36 hour time-point, TIMP1 levels decreased to 92,769 pg/mL and 99,702 pg/mL in dosing groups 200 and 500 mg/kg, respectively. Concentration of the protein continued to decrease to as low as 47,505 pg/mL and 51,476 pg/mL in dosing groups 200 and 500 mg/kg, respectively, at the 48 hour time-point. A two-way analysis of variance (ANOVA), which required a Box-Cox transformation to remedy a violation of equal variance, showed that TIMP1 levels between time points 48, 96, 108 and 120 hours did not differ in any of the three dosing groups. This suggests that a plateau in the excretion of the biomarker was reached and that TIMP1 does not exhibit a biphasic expression profile as initially thought. Statistical analysis of BACC adjusted TIMP1 values (Figure 16) indicated that there were no significant differences between 200 and 500 mg/kg dose groups. As discussed previously (Section 2.1), the profile data was normalized and graphed using either urine volume-weighted (Figure 14), creatinine-adjusted (Figure 15), or BACC baseline adjustments (Figure 16) for comparisons to TIMP1 published data.
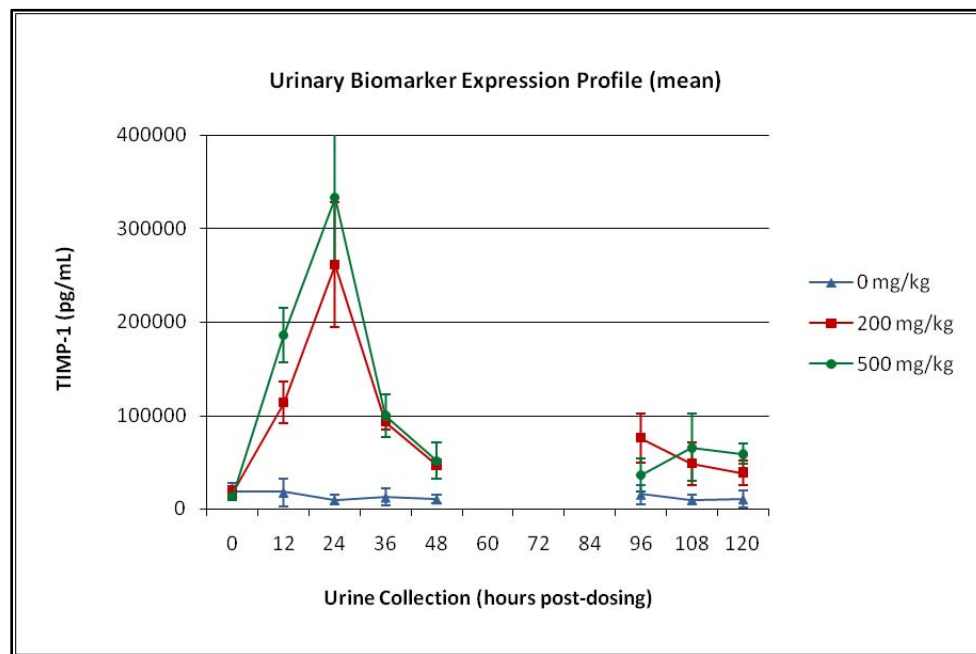
Figure 13: **Measurement of urinary TIMP1 versus time after D-serine administration (Study 2U)**
No TIMP1 analysis was done on samples taken between 48-96 hrs

24
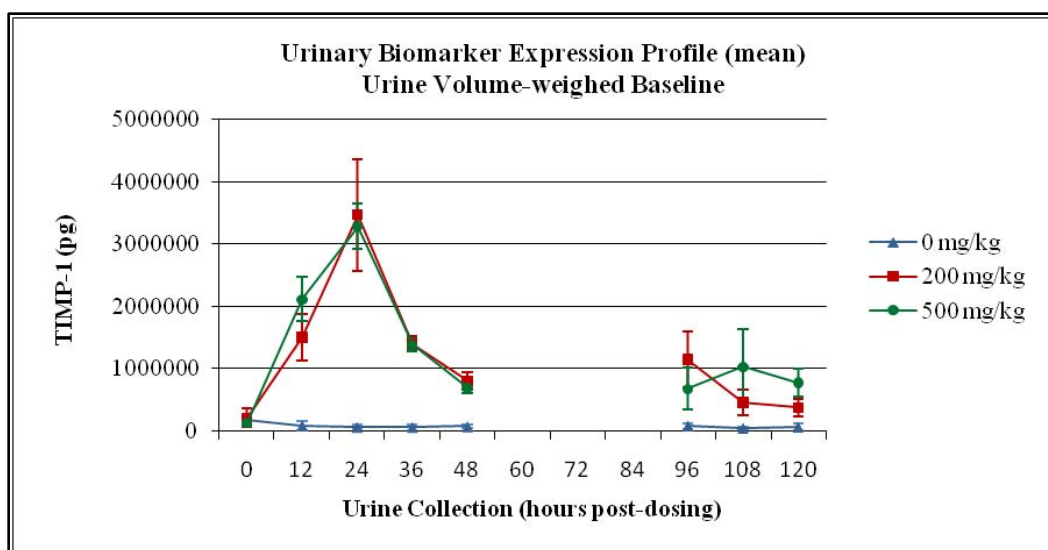
Figure 14: Measurement of urinary TIMP1 (urine volume-weighted) versus time after D-serine administration (Study 2U)
No TIMP1 analysis was done on samples taken between 48-96 hrs
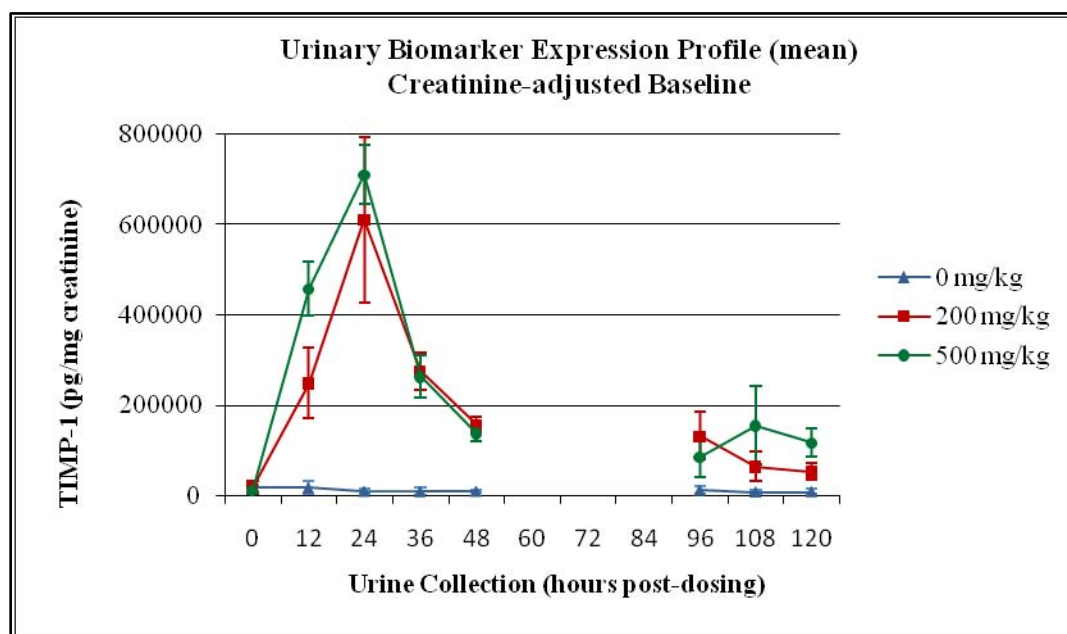
Figure 15: Measurement of urinary TIMP1 (ng/mg creatinine) versus time point after D-serine administration (Study 2U)
TIMP1 was not analyzed in samples between 48 and 96 hours

25
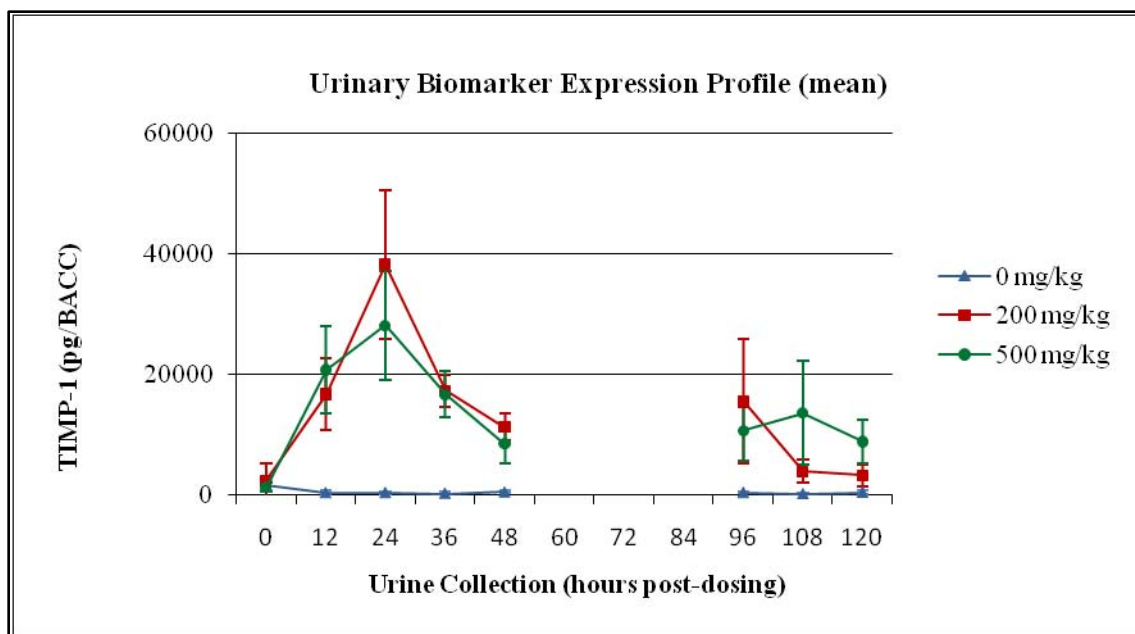
Figure 16: Measurement of urinary TIMP1 (BACC adjusted) versus time point after D-serine administration (Study 2U)
TIMP1 was not analyzed in samples between 48 and 96 hours
Examination of TIMP1 in serum from Study 2S

A two-way analysis of variance (ANOVA) showed that TIMP1 expression did not differ significantly between the 12 and 24 hour time points of dosing groups 0, 200 and 500 mg/kg. In the control group, the biomarker reached 5772 pg/mL and 5171 pg/mL (excluding 1 outlier) at the 12 and 24 hour time points, respectively (Figure 17). In the 200 mg/kg dosing group, TIMP1 reached 7600 pg/mL and 6562 pg/mL at the 12 and 24 hour time points, respectively. In the 500 mg/kg dosing group, TIMP1 reached 8485 pg/mL and 8142 pg/mL at the 12 and 24 hour time points, respectively. A one-way ANOVA between dosing groups on pooled 12 and 24 hour data showed no significant differences in the expression of TIMP1 between the control and either dosing group or between the 200 and 500 mg/kg dosing groups. One 'outlier' value was seen in the 0 mg/kg control group, and the data from this test fell within our standard accepted CV range. This high TIMP1 value from this single animal may have been due to illness or injury not found in the other group animals.
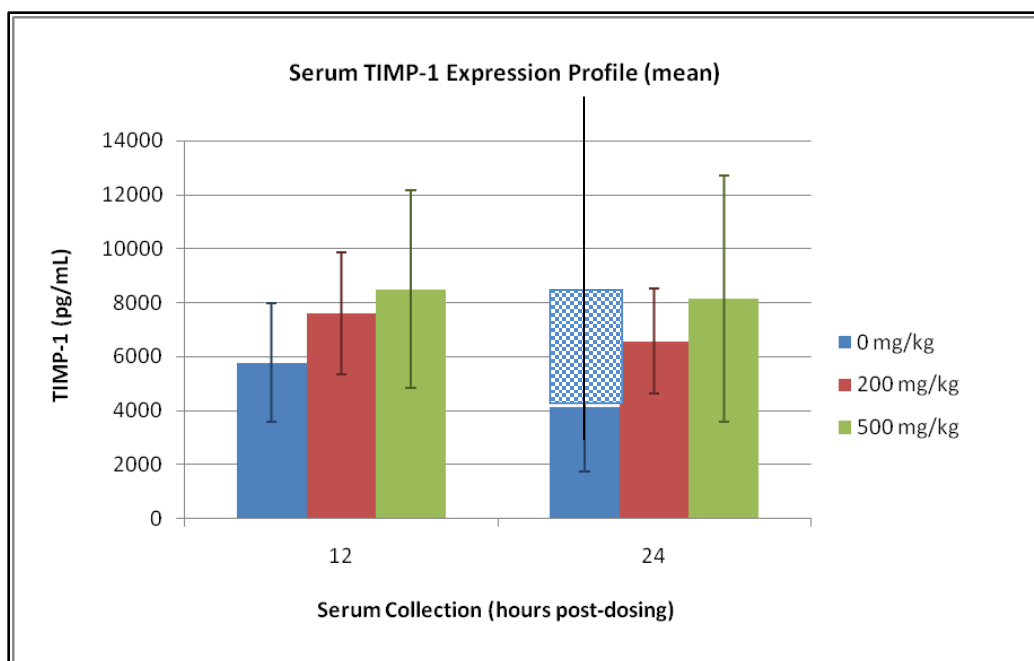
Figure 17: **Measurement of serum TIMP1 by ELISA at 12 h and 24 h after D-serine administration**
Levels in the control group at 24 hrs indicate both total (solid + hatched)
and without outlier value (solid)

While the changes seen do reflect dose levels, the lack of significance in the changes of the TIMP1levels between dose groups put into question the use of TIMP1 as a serum biomarker for low level kidney injury. It may be possible that by using an assay capable of greater sensitivity, the tested levels may reflect differences that are statistically significant. Serum TIMP1 has been shown to have potential as a prognostic biomarker to gastric cancer (Wang et al., 2006) and invasive colorectal cancer (Pellegrinni et al., 2000) in the human. However, in this nephrotoxin model using our current ELISA testing procedure, TIMP1 response in the serum does not provide a significant biomarker response to be considered as a punitive serum biomarker for sub-clinical kidney injury.

The acquired data *does* indicate that TIMP1 works well as a urinary biomarker to subclinical kidney injury in our model. The fairly fast and strong signal in the urine in response to kidney injury is understandable as TIMP1 has been shown to be produced by the interstitial cells in the kidney (Duymelinck et al., 2000) putting the protein in proximity for fast deposition into the urine. BACC adjusted TIMP1 values (Figure 16) indicate a strong response at 24 hrs post-dose.

Interestingly, BACC normalization of this data indicates a stronger TIMP1 response in the 200 mg/kg dose group versus the 500 mg/kg dose group, whereas other normalization methods indicate the reverse (Figure 14 and Figure 15). However, in all cases the standard deviations from one dose group falls within the peak level of biomarker response of the other, so

the apparent stronger response of the 200 mg/kg may not be significant. The initial nephrotoxin data indicated a much smaller response at 96 hrs, but further studies determined that this increase was minor in nature and not reflective of any biphasic response of TIMP1.

In Rached et al., 2008, an examination of TIMP1 expression in the kidney of Fischer 344 rats after treatment with the nephrotoxin ochratoxin A (OTA) at 210 ug/kg indicated that expression was not significantly altered upon OTA exposure. Immunohistopathology of the kidney indicated that the TIMP1 protein *was* detected in the proximal tubules of both the kidney cortex and the outer stripe of the outer medulla (OSOM), as well as in the tubular lumina (also seen by Eddy et al., 1995). These protein expression changes in the kidney were shown to be very small in this model, although in other kidney injury models TIMP1 expression and protein levels (via immunohistochemistry) were seen to increase (Eddy et al., 1995). It was proposed by Rached that since TIMP1 protein was not seen in the tubulointerstitial space in this nephrotoxin model, TIMP1 may have multiple roles in kidney injury damage, depending upon mode of damage, and at least in this nephrotoxin model may be involved in proximal tubular cell proliferation in response to damage. The increase in TIMP1 seems to be an interstitial cellular response to toxin insult. However, it is not clear at this point if further damage leads to necrosis and the release of cellular contents into the tubules, or if the urinary increase occurs from a normal diffusion mechanism and due to both the increase in TIMP1 cellular expression combined with an increase in interstitial cells lining dilated/injured tubules (Duymelinck et al., 2000).

2.3 Clusterin

2.3.1 Clusterin Summary

Clusterin is strongly supported as a urinary biomarker to kidney damage in many published papers using both human and animal models, and therefore was examined for potential responses to renal damage in a nephrotoxin model. The urine expression profiling of clusterin was accomplished in Fischer 344 rat model using the nephrotoxin D-serine administered at levels to induce subclinical kidney damage. Male Fischer 344 rats were administered intraperitoneally with 0, 5, 20, 50, 200, or 500 mg/kg D-serine in 0.9 percent saline, with control animals injected with saline alone. In animal Study 1, urine was collected pre-dose and at 24, 48, 72 and 96 hours post-dose, frozen within 1 hour of end point collection, and later assayed for clusterin by ELISA. Levels of kidney damage were verified by histopathological examination. Clusterin levels remained constant for 0, 5, 20, and 50 mg/kg D-serine throughout the time course. However, for animals in the 200 and 500 mg/kg D-serine groups, significant increases were observed with a peak at 24 hours post-dose. Clusterin levels dropped near pre-dosing levels at 72 hours post-dose. However, marked increases in Clusterin levels were again observed at 96 hours post-dose. A second time-course experiment was conducted in order to confirm the biphasic nature of the biomarker response. In Study 2U, doses of 0, 200 or 500 mg/kg D-serine were used as previously. In Study 2U, however, urine samples were collected at 12 hour intervals using a longer 168 hour period. Significant increases in clusterin were again observed at 24 hours post-dose, with levels decreasing near baseline levels by 48 hours. As previously observed, an increase in clusterin levels was observed in a secondary peak at 96 to 120 hours post-dose, with levels reaching the concentrations observed at 24 hours post-dose. This data confirms the biphasic nature of the clusterin expression profile. In Study 2S, serum samples were assayed by ELISA for clusterin. Blood was collected at 12 and 24 hours post-dose in control animals and animals dosed with 200 mg/kg and 500 mg/kg of D-serine. No statistically significant differences in clusterin levels were observed between the 12 and 24 hour time points of dosing groups 0, 200 and 500 mg/kg. A one-way ANOVA between dose groups on pooled 12 and 24 hour data revealed no significant differences in the expression of clusterin between the control and either dosing group, but demonstrated that the 200 mg/kg dose group was significantly higher than the 500 mg/kg dose group. This report indicates that clusterin protein, in urine, is a valid biomarker for the examination of subclinical kidney damage in a nephourotoxin model in the rat. The clusterin response is biphasic with an initial response at 24 hours followed by a response of equal intensity at 96 hours post-dose. As other published data indicates a response in both disease and toxin models, clusterin is a prime candidate for inclusion on any biomarker panel for monitoring kidney health in a field testing situation.

2.3.2 Clusterin Introduction

Clusterin is a 80 kDal glycoprotein with a wide breadth of distribution in various tissue and body fluids with diverse physiological functions within the organism (Jones et al., 2002). In humans, two isoforms are produced: a glycosylated secreted heterodimer (sCLU) and a truncated nuclear form (nCLU). In the blood, the heterodimer sCLU form consists of two non-identical subunits with ~30% of the dimer mass is due to N-linked carbohydrates. The sCLU dimer is

linked by 5 disulfide bridges (Tschopp et al., 1994; Jenne et al., 1992).   It is the glyscosylated isoform which has been examined in this study for its response to cell injury.

Clusterin levels have been shown to increase in response to many disease states (ageing, cancer, vascular damage, neuron degeneration, diabetes) which represent increased oxidative stress which in turn promotes cell death (Strocchi et al., 2006). While its response was initially examined in cancer and aging, clusterin was identified as a possible urinary biomarker to renal injury by nephrotoxins early as 1992 (Aulitzky et al., 1992). In addition to nephrotoxin injury, clusterin responds to several other renal diseases such as congenital obstructive nephropathy (Chevalier, 2004), polycyctic kidney disease (Gallagher et al., 2002) and AKI (Yoshimura et al., 1996). In the rat model, clusterin levels has been shown to increase in bilateral renal ischemia (Hidaka et al., 2002) as well as in unilateral ureteral obstruction (Ishii et al., 2007).

The modulation of urine and blood clusterin in the rat has been examined using the nephrotoxins gentamicin (Aulitzky et al., 1992; Eti et al., 1993; Sieber et al., 2009) and ochratoxin (Rached et al., 2008). While modeled as a chronic rather than an acute exposure, such studies indicated that clusterin occurs in response to tubule cell damage. These studies also indicated clusterin changes at 4 days or greater, but did not examine urinary changes from 0 to 96 hours in a single acute exposure. A study examining puromycin aminonucleoside toxicity in an acute model in the rat concluded that this nephrotoxin induced clusterin expression primarily at the tubules of the cortex and medulla (Correa-Rotter et al., 1998).

2.3.3 Clusterin Methods, Assumptions, and Procedures

Urine and Serum Collection

More detailed descriptions of the animal studies are described in *Biomarkers of Exposure to Toxic Substances, Vol 1* (DelRaso et al., Sep 2009). However, for clarity of this section, we include brief descriptions of each study.

D-serine Study 1

Male Fischer 344 rats were dosed with 5, 20, 50, 200 or 500 mg/kg D-serine in saline, whereas control animals were dosed with saline alone. Five animals were included in each dosing group with the exception of the 200 mg/kg group in which only four animals were included. Urine was collected in metabolic cages from animals prior to dosing and at 24, 48, 72 and 96 hours post-dose. One percent sodium azide, a bacteriostatic preservative, was added to each sample, and urine was frozen at -20°C within 1 hour of end point collection.

D-serine Study 2U (urine)

Male Fischer 344 rats were dosed with 200 or 500 mg/kg D-serine in saline, whereas control animals were dosed with saline alone. Five animals were included in each dosing group with the exception of the control group in which only four animals were included. Urine was collected from animals prior to dosing and at 12-hour intervals up to 168 hours post-dose. Protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) equal to 1/100 of the urine volume was added, and urine was frozen immediately at -20°C before being moved into a -80°C freezer within 1-7 days.

D-serine Study 2S (serum)

Male Fischer 344 rats were dosed with 200 or 500 mg/kg D-serine in saline, whereas control animals were dosed with saline alone. Ten animals were included in each dosing group. Five of the ten animals were sacrificed 12 hours post-dose. The remaining five animals were sacrificed 24 hours post-dose. Blood was collected from the inferior vena cava.

Validation of Clusterin ELISA for evaluations in rat urine.

ELISA Kit Evaluation1

Enzyme-linked immunosorbent assays were performed using BioVendor rat Clusterin ELISA kits (BioVendor Laboratory Medicine, Inc., Czech Republic). For one urine sample expected to have measurable levels of clusterin, 8-point, and 2-fold serial dilutions were performed, beginning at 1:20 and ending at 1:2560. Additionally, the 8-point, 2-fold serial dilution was performed for one urine sample expected to have baseline levels of clusterin.

ELISA Kit Evaluation 2

For four urine samples expected to have measurable levels of clusterin, 6-point, and 2-fold serial dilutions were performed, beginning at 1:4 and ending at 1:128. In addition, reference standard material at concentrations of 0 and 32 ng/mL of clusterin were spiked into a baseline urine sample diluted 1:4, 1:20 and 1:200.

ELISA Kit Evaluation 3

One urine sample expected to have measureable levels of clusterin was diluted 1:2, 1:4, 1:8 and 1:16. In addition, clusterin was spiked into a baseline urine sample at 0, 6, 20 and 64 ng/mL. The urine was diluted 1:10.

Urine and Serum Clusterin ELISA Analyses

Enzyme-linked immunosorbent assays were performed as described in the BioVendor rat Clusterin ELISA kit protocol. First, urine samples were diluted 1:4 and serum samples were diluted 1:2000 with dilution buffer. One hundred microliters of diluted sample were added to the wells of 96-well microtiter plates coated with anti-rat clusterin polyclonal antibody and incubated for 1 hour at room temperature with shaking at 300 rpm. Plates were washed three times with wash buffer. One hundred microliters of biotinylated anti-rat clusterin antibody diluted in biotin-antibody diluent were added and incubated for 1 hour at room temperature with shaking at 300 rpm. Wells were washed three times, 100 uL of streptavidin-HOURP conjugate was added to the wells, and plates were incubated for 30 minutes at room temperature with shaking at 300 rpm. Wells were washed three times, 100 uL of TMB substrate solution (3,3', 5,5'-tetramethylbenzidine) was added, and plates were incubated 10 minutes at room temperature in the dark. Reactions were stopped by the addition of 100 uL of 0.2 M sulfuric acid. The absorbance (optical density [OD]) was measured at 450 nm with a microplate reader (Molecular Devices SpectraMax M2[e]).

Statistical Analysis

All data were the average of duplicate determinations. The results were expressed as mean values ± standard deviations (SD). Statistical methodology included analysis of variance (H0: $\mu_1 = \mu_2 = \dots = \mu_k$ versus H_a: $\mu_i = \mu_j$). When a significant difference was found at $\alpha = .05$, Tukey's multiple comparisons were performed to identify which dosing groups differed significantly. To ensure validity of the analysis of variance, the Kolmogorov-Smirnov test of normality and Bartlett's test for equal variances were performed on the residuals. Where normality was not justified, the Kruskal-Wallis test was performed in lieu of the analysis of variance, followed by Tukey's multiple comparisons as appropriate. When the assumption of equal variances failed, transformations were performed to stabilize the variance.

2.3.4 Clusterin Results and Discussions

Clusterin ELISA Kit Performance Evaluations

The BioVendor rat Clusterin ELISA kit is validated for use with rat serum but not urine. Therefore, the kit was evaluated by three independent analyses in order to assess the kit's capability of detecting clusterin in rat urine. First, the kit's capability of detecting clusterin from in-house rat urine samples expected to have measurable levels of the protein was assessed. Second, the linearity of the results obtain from multiple dilutions of a urine sample was assessed. Third, the recovery efficiency of clusterin from the urine matrix was assessed through a spike and recovery experiment.

ELISA Kit Evaluation 1

The kit insert recommended a starting dilution of 1:2000 for samples. The first evaluation was designed to assay urine samples expected to have either baseline levels or relatively high levels of clusterin at multiple dilutions to establish the appropriate sample dilution. Dilutions were chosen to include the manufacturer's recommended dilution factor and to assess how low urine samples could be diluted without losing dilutional linearity.

For the baseline urine sample, all ODs were below the range of the standard (Figure 18), thus below the limit of detection (data not shown). The other sample, expected to have relatively high levels of clusterin, had ODs within the range of the standard for dilution points 1:20, 1:40, 1:80, and 1:160. The remaining points were out of the standard range (low end). The dilutional linearity for points 1:20 through 1:160 was demonstrated, with an intra-assay CV of only 3.03 percent (Table 9). Results of both QCs fell within the acceptable range established by the manufacturer (data not shown).

The ODs of the sample expected to have relatively high levels of clusterin suggested the protein can be detected in urine. The dilution experiment indicated that, contrary to the manufacturer's recommendation, a 1:2000 dilution of samples is not required. Instead, clusterin was measureable in urine at dilutions ranging from 1:20 to 1:160. A second dilution experiment was performed to determine whether samples can be diluted less without losing dilutional linearity. The evaluation included a spike and recovery experiment in which 0 and 32 ng/mL were spiked into urine diluted 1: 4, 1:20 and 1:200.

Table 9: **Clusterin analysis of test animal urine at dilution factors of 20, 40, 80, 160, 320, 640, 1280 and 2560**

* Outside of the range of the standard.

OD Value	Result (ng/mL)	Mean Result (ng/mL)	Std Deviation	Percent CV	Dilution Factor	Adjusted Result (ng/mL)	Mean Adjusted Result	Intra-Assay percent
0.921	31.743							
0.947	32.971	32.357	0.868	2.7	20	647.143		
0.565	16.279							
0.522	14.592	15.436	1.193	7.7	40	617.429	645.560	3.03
0.352	8.464							
0.339	8.027	8.245	0.309	3.7	80	659.612		
0.217	4.234							
0.208	3.992	4.113	0.171	4.2	160	658.073		
*0.170	NA							
*0.159	NA		NA		320	NA		
*0.125	NA							
*0.129	NA		NA		640	NA	NA	NA
*0.106	NA							
*0.107	NA		NA		1280	NA		
*0.097	NA							
*0.096	NA		NA		2560	NA		

Figure 18: Clusterin Standard Curve

ELISA Kit Evaluation 2

The dilutional linearity for points 1:4 to 1:128 was demonstrated, with intra-assay CVs ranging from 4.89 to 17.93 percent (Table 10). The recoveries of clusterin from urine diluted 1:4, 1:20 and 1:200 were 57.3 percent, 70.1 percent and 72.8 percent, respectively. The kit only displayed a recovery of approximately 67 percent (Table 11). However, this recovery was based on a single concentration of 32 ng/mL of clusterin spiked into urine.

Based on evaluations 1 and 2, the lowest recommended starting dilution of urine to be assayed with the BioVendor rat Clusterin ELISA kit is 1:4. Recoveries of clusterin from spiked urine ranged from 57-73 percent at the acceptable dilutions. A third dilution experiment was performed in order to assess whether urine samples can be diluted as low as 1:2. In addition, a spike and recovery experiment was performed. Clusterin was spiked into a baseline urine sample at 0, 6, 20 and 64 ng/mL. The urine was diluted 1:10.

ELISA Kit Evaluation 3

It should be noted CVs for the points of the standard curve as well as test sample replicates exceeded 20 percent. However, the results for the QCs were within the acceptance range specified by the manufacturer and the R^2 for the reference standard curve was 0.996.

34

The intra-assay CV between dilution points 1:2 to 1:16 was 28 percent with the 1:2 dilution result being furthest from the mean (Table 10). Upon exclusion of the result obtained from the 1:2 dilution, the intra-assay CV decreased to less than 15 percent, suggesting urine samples cannot be diluted as low as 1:2. The recoveries of clusterin from a baseline urine sample diluted 1:10 and spiked with 6, 20 and 64 ng/mL were 100 percent, 91 percent and 90 percent, respectively (Table 11). Based on all three kit evaluations, the lowest recommended starting dilution of urine to be assayed with the BioVendor rat Clusterin ELISA kit is 1:4.

Table 10: Clusterin Dilutional linearity between a urine sample diluted 1:2, 1:4, 1:8 and 1:16.

Dilution Factor	Mean OD	Adjusted Result (ng/mL)	Duplicate percent CV	Mean	Percent CV
2	1.353	110.012	4.7		
4	1.147	174.101	4.4	175.610	28.36
8	0.731	188.132	11.8		
16	0.499	230.201	3.4		

Table 11: Spike and recovery of 0, 6, 20 and 64 ng/mL clusterin.
Clusterin (reference standard material) was aliquoted into a 1:10 dilution of baseline urine sample

Spiked concentration (ng/mL)	Mean OD	Mean Result (ng/mL)	Replicate CVpercent	Adjusted Result (spike-4.509 ng/mL)	Urine Dilution	Percent Recovery
0	0.169	4.509	6.8	0	1:10	NA
6	0.385	10.556	15.7	6.047	1:10	100.8
20	0.708	22.714	30.2	18.205	1:10	91.0
64	1.459	61.941	34.6	57.432	1:10	89.7

Clusterin Profiling in Urine

D-serine Study 1

The nephourotoxin D-serine was used to induce subclinical kidney damage at the proximal straight tubules in order to evaluate clusterin as an early predictor of nephourotoxicity. Doses of 20, 50, 200 or 500 mg/kg D-serine were administered intraperitoneally in 0.9 percent saline, whereas control animals were dosed with saline alone. Urine samples were collected at 24 hours intervals in a 96 hour period. Clusterin levels in the urine were quantitated by sandwich ELISA and the biomarker expression profile determined.

Significant biomarker profile changes in clusterin were seen in urine only in the 200 and 500 mg/kg D-serine dose groups. No significant profile changes were seen at the 5, 20 or 50 mg/kg D-serine dose groups (data not shown). Kidney histopathology data and clinical

chemistry data (TBIL, CREA, BUN, and Total Protein) confirmed the initiation of kidney damage around the 200 mg/kg dose (discussed in DelRaso et al., Sep 2009). The biomarker profile peaked at 24 hours, reaching 845 ng/mL and 753 ng/mL in dose groups 200 and 500 mg/kg, respectively (Figure 19. Measurement of urinary clusterin by ELISA over time after D-serine administration (Study 1)) with, dose groups 200 and 500 mg/kg were significantly higher than the control group. The 200 and 500 mg/kg dose groups were not significantly different from each other ($p<0.05$) at 24 hours. At the 72 hour time-point, clusterin levels decreased to as low as 306 ng/mL and 133 ng/mL in dosing groups 200 and 500 mg/kg, respectively. Levels were found to increase again at the 96 hour time point with clusterin concentrations reaching 1343 ng/mL and 711 ng/mL in dosing groups 200 and 500 mg/kg, respectively. No plateau was observed in the excretion of clusterin during the 96 hour time-course. Instead, clusterin may exhibit a biphasic expression profile. A longer time-course experiment extending to 120 hours was conducted to clarify any relevancy and extent of the 96 hour peak.
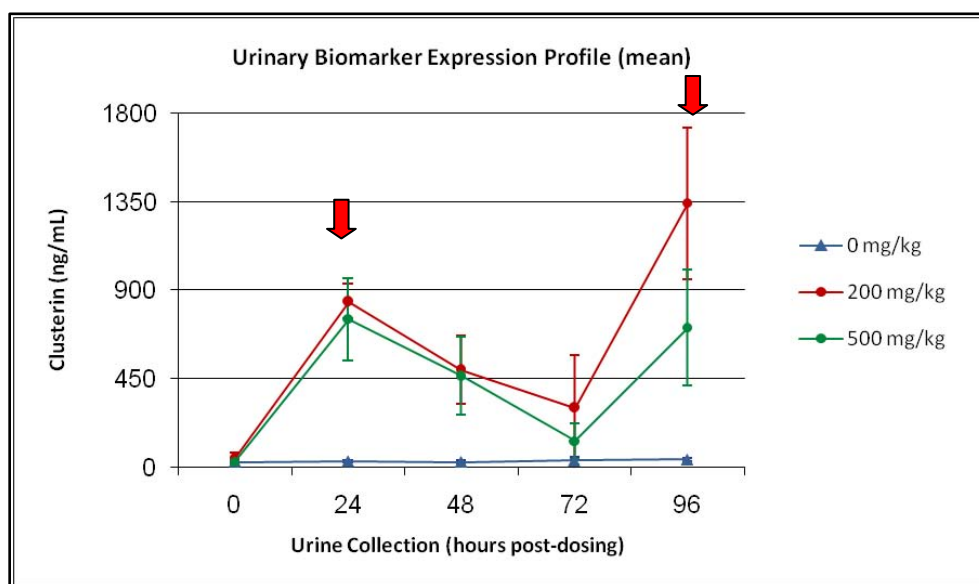
Figure 19. Measurement of urinary clusterin by ELISA over time after D-serine administration (Study 1)
Biphasic response indicated by red arrows

D-serine Study 2U

A second time-course experiment was conducted in order to clarify the relevancy of the increase in clusterin concentration at the 96 hour time point as well as to confirm that the potential biomarker marker peaks at 24 hours. This second study, Study 2U, extended the post-dose collection period to 168 hours, with urine samples collected every 12 hours. Clusterin was quantitated by h ELISA for the time periods 0-48 hours and 96 to 120 hours, to determine if the 96 hour peak response was an artifact or a valid signal.

As previously observed in the original time-course experiment, significant biomarker profile changes in clusterin were seen in urine in the 200 and 500 mg/kg D-serine dose groups. Kidney histopathology data and clinical chemistry data (TBIL, CREA, BUN, and Total Protein)

confirmed subclinical kidney damage at the 200 and 500 mg/kg doses (discussed in DelRaso et al., Sep 2009). The urine profile of the biomarker peaked at 24 hours, reaching 1343 ng/mL and 1314 ng/mL in dosing groups 200 and 500 mg/kg, respectively (Figure 20: Measurement of urinary clusterin by ELISA versus time after D-serine administration (Study 2U)).   At peak expression, dosing groups 200 and 500 mg/kg were significantly larger than the control group. However, the 200 and 500 mg/kg dosing groups were not significantly different from each other (p<0.05). At the 36 hour time-point, clusterin levels decreased to 505 ng/mL and 641 ng/mL in dosing groups 200 and 500 mg/kg, respectively. The concentration of the protein continued to decrease to as low as 160 ng/mL and 276 ng/mL in dosing groups 200 and 500 mg/kg, respectively, at the 48 hour time-point. A two-way analysis of variance (ANOVA), which required a Box-Cox transformation to remedy a violation of equal variance, showed that clusterin levels between time-points 48 and 96 hours were significantly different in both the 200 mg/kg and 500 mg/kg dosing groups. No significant differences were observed between time-points 96 and 108 hours or time-points 108 and 120 hours. As discussed previously (Section 2.1), the profile data was normalized and graphed using either urine volume-weighted (Figure 21), creatinine-adjusted (Figure 22), or BACC baseline adjustments (Figure 23) for comparisons to Clusterin published data.
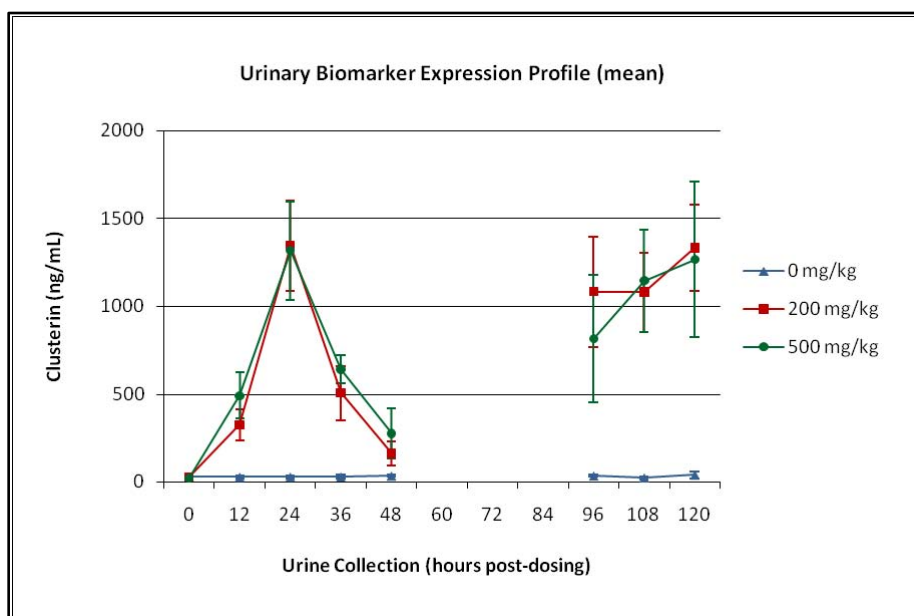
Figure 20: **Measurement of urinary clusterin by ELISA versus time after D-serine administration (Study 2U)**
Clusterin was not assayed in samples taken between 48 to 96 hours
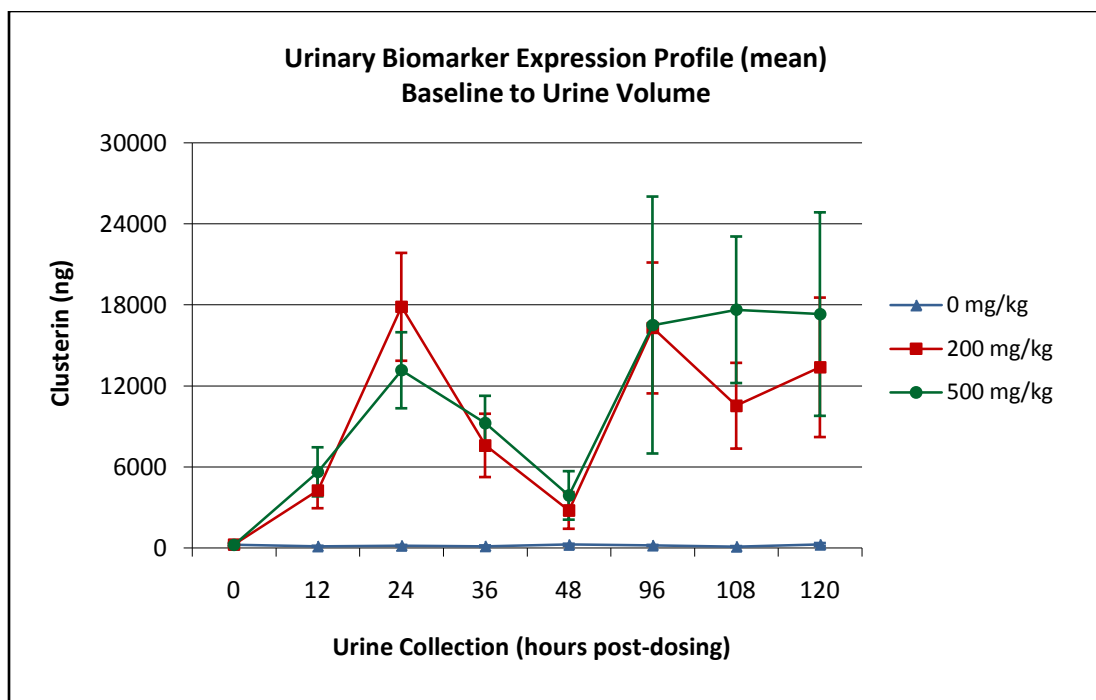
Figure 21: **Measurement of urinary clusterin by ELISA versus total urine volume/time point after D-serine administration (Study 2U)**
Urine volume-weighted clusterin values were used.
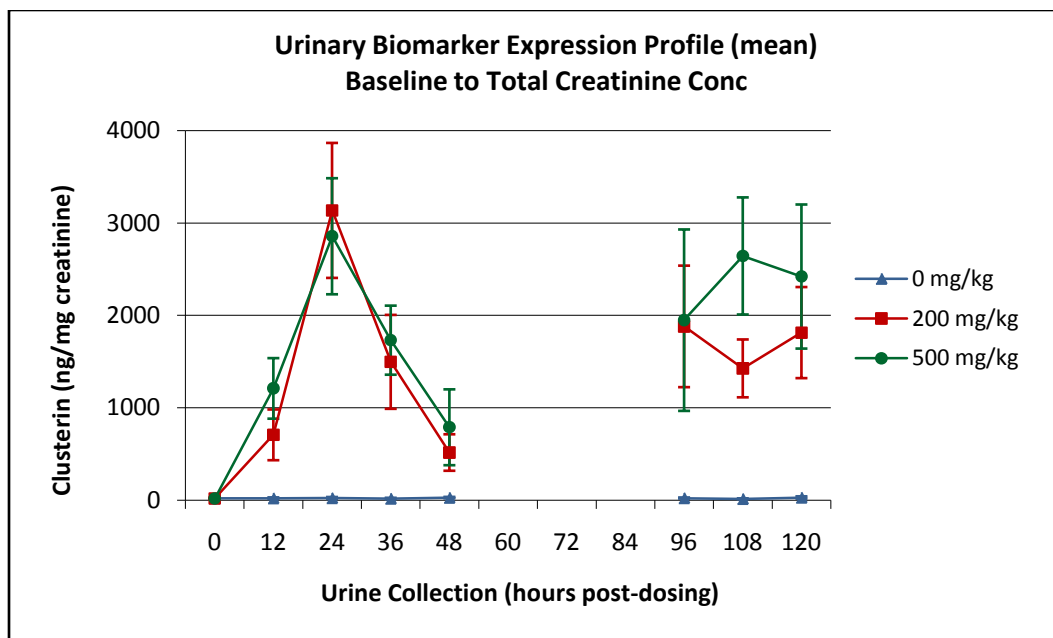
Figure 22: **Measurement of urinary clusterin (ng/mg creatinine) versus time point after D-serine administration (Study 2U)**
Creatinine-adjusted clusterin values were used. Clusterin was not assayed in samples taken between 48 to 96 hours
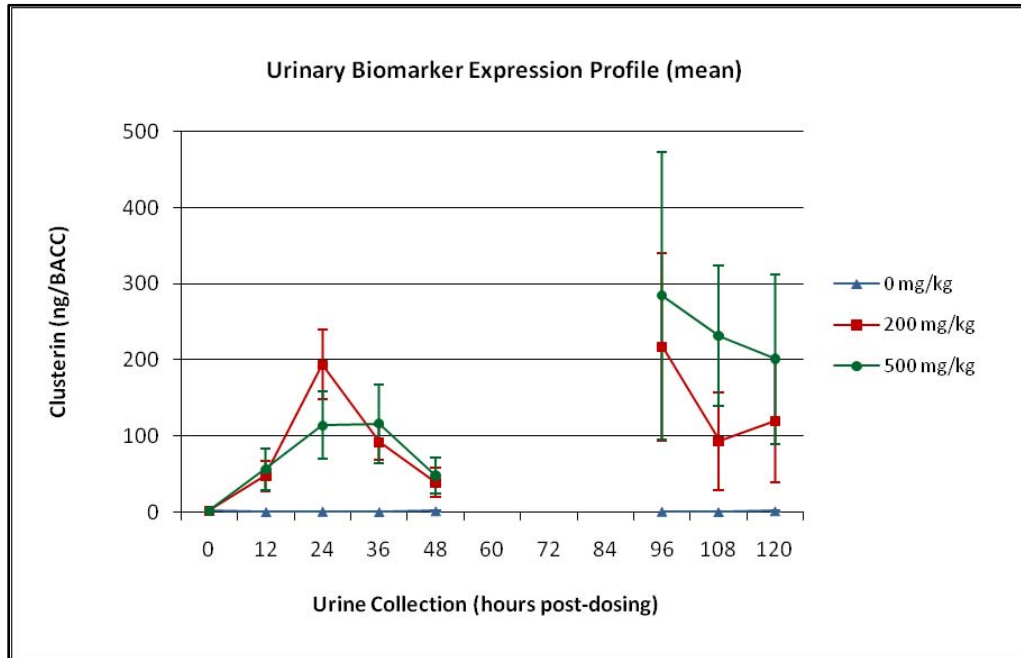
Figure 23: Measurement of urinary Clusterin (BACC adjusted) versus time point after D-serine administration (Study 2U)
Clusterin was not assayed in samples taken between 48 to 96 hours

Clusterin Profiling in Serum

D-serine Study 2S

A two-way analysis of variance (ANOVA) showed that serum clusterin expression did not differ significantly between the 12 and 24 hour time points of dosing groups 0, 200 and 500 mg/kg. In the control group, the biomarker reached 73,767 ng/mL and 79,918 ng/mL at the 12 and 24 hour time points, respectively (Figure 24). In the 200 mg/kg dosing group, clusterin reached 91,852 ng/mL and 75,127 ng/mL at the 12 and 24 hour time points, respectively. In the 500 mg/kg dosing group, clusterin reached 64,639 ng/mL and 67,670 ng/mL at the 12 and 24 hour time points, respectively. A one-way ANOVA between dosing groups on pooled 12 and 24 hour data showed no significant differences in the expression of clusterin between the control and either dosing group, but showed that the 200 mg/kg dosing group was significantly higher than the 500 mg/kg dosing group.
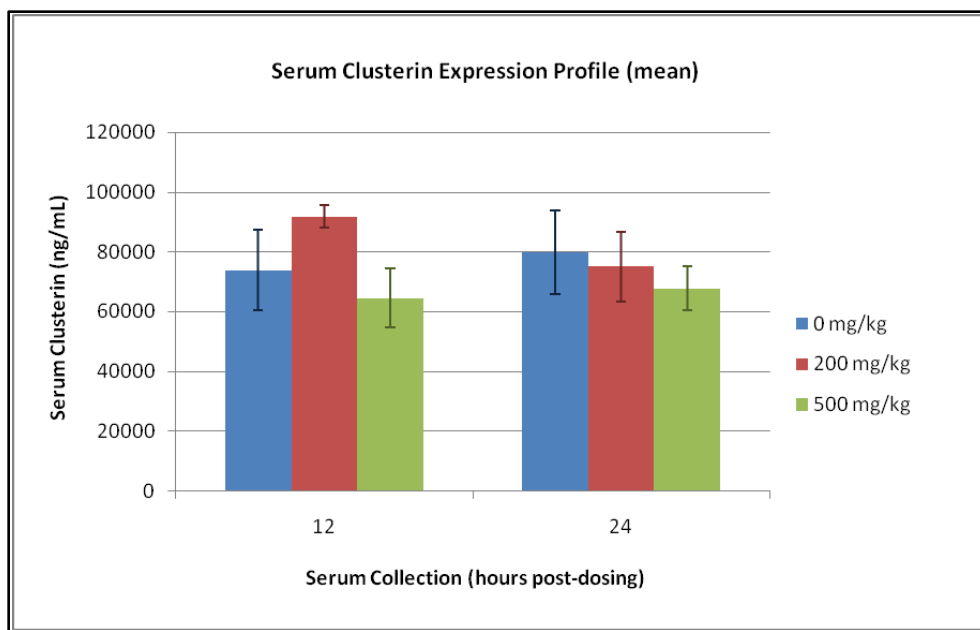
Figure 24: **Measurement of serum clusterin by ELISA at 12 h and 24 h after D-serine administration**

2.4   Retinol Binding Protein 4 (RBP4)

2.4.1 RPB4 Summary

An examination of current literature revealed retinol binding protein 4 (RBP4) as a potential biomarker predictive of acute, chronic, chemically-induced, or disease-induced kidney damage. The protein expression profile of RBP4 in urine was examined in a time dose response using several concentrations of the known nephrotoxin D-serine. Male Fischer 344 rats were dosed with 5, 20, 50, 200, or 500 mg/kg D-serine in saline, whereas control animals were dosed with saline alone. Urine was collected from animals prior to dosing and at 24, 48, 72 and 96 hours post-dosing and frozen within 1 hour of end point collection. The samples were assayed for RBP4 by ELISA. Clusterin levels remained constant for control animals and in animals dosed with 5, 20, and 50 mg/kg D-serine throughout the time course. However, for animals dosed with 200 and 500 mg/kg D-serine, significant increases were observed with a peak at 24 hours post-dose. RBP4 levels returned to baseline levels at 96 hours post-dose. A second experiment was conducted in order to confirm the peak response timesof the protein biomarker. Again, D-serine was used to induce sub-clinical kidney damage at the proximal straight tubules using 0, 200 or 500 mg/kg D-serine administered intraperitoneally in 0.9% saline. Control animals were dosed with saline alone. Urine samples were collected at 12 hour intervals in a 168 hour period and frozen immediately. The potential RBP4 was quantitated by sandwich ELISA. As previously observed, maximul urinary RBP4 expression was observed at 24 hours post-dose, with levels decreasing 36-48 hours post-dose. In addition to assaying urine samples, serum samples were also examined for RBP4 experssion upon nephrotoxin exposure. Blood was collected at 12 and 24 hours post-dosing in animals dosed with0, 200 and 500 mg/kg of D-serine. No statistically significant differences in RBP4 levels were observed between the 12 and 24 hour time points of dosing groups 0, 200 and 500 mg/kg. A one-way ANOVA between dosing groups on pooled 12 and 24 hour data showed no significant differences in the expression of RBP4 between the 200 and 500 mg/kg dosing groups. Interestingly, ANOVA analysis indicated that the control group was significantly higher than both dosing groups in the serum. Therefore, these studies indicate that RBP4 is a potential biomarker in the urine indicative of subclinical renal injury in a nephrotoxin model, but RBP4 in the blood may actually decrease upon toxin exposure.

2.4.2 RPB4 Introduction

Retinol binding protein 4 (RBP4) is a 21 kDal protein synthesized in the liver and secreted from liver and adipose tissue. There are several isoforms of RBP4: apo-RBP4, holo-RBP4, and two forms truncated at the C-terminus, RBP4-L and RBP4-LL, although it is not clear at this time what or if the role of each isoform plays in RBP4 function or biomarker expression.

RBP4 is known to transport retinol in the blood to insure regulation in the plasma, and exists in plasma as a complex with retinol and transthyretin (Naylor et al., 1999). The mass of this three component complex prevents its filtration from the kidney. However, once retinol is released, the apoRBP4 is quickly filtered out the kidney glomerulus then reabsorbed into the proximal tubule cells to be catabolized (Kiernan et al., 2002; Goodman, 1980). Thus, the primary region of synthesis is in the liver, while the kidney is the location of RBP4 catabolism. Due to these separate locations of synthesis and degradation, RPB4 levels have been examined for

expression modulation in both kidney (Ziegelmeier et al., 2007) and liver degenerative diseases (Tacke et al., 2008). RBP4 has been shown to decrease in serum in patients with chronic liver disease and cirrhosis, while increasing in liver degenerating diseases such as Hepatitis C virus and alcoholic steatohepatitis (Petta et al., 2008).

Since the kidney is involved in the catabolism of RBP4, renal injury such as chronic kidney disease results in elevated RBP4 in the plasma (Frey et al., 2008). RBP4 has also been examined in serum from diabetic and non-diabetic patients on chronic hemodialysis versus controls (Ziegelmeier et al., 2007). This study indicates that RBP4 levels correlate with c-reactive protein in serum as well as renal function and inflammation, although these findings are in dispute (Papavasileiou et al., 2008). RBP4 has also been examined in the plasma of Type 2 Diabetes mellitus patients with nephropathy and these data demonstrate that RBP4 levels were up to 3 fold higher in patients with end-stage renal disease (Masaki, 2008). An examination of urinary excretion of RBP4 in a study examining low birth weight infants and HIV-associated renal diseases demonstrated that RBP4 levels in the urine may be related to impaired tubular re-absorption of RBP4 (Nagl et al., 2009; Soler-García et al., 2009). At this time, there are no reported studies examining RBP4 in a nephrotoxin model, therefore an examination of its uses as a urinary marker for toxin-induced renal injury would expand the detection capabilities of this protein.

2.4.3 RPB4 Methods, Assumptions, and Procedures

Urine and Serum Collection

More detailed descriptions of the animal studies are described in *Biomarkers of Exposure to Toxic Substances, Vol 1* (DelRaso et al., Sep 2009). However, for clarity of this section, we include brief descriptions of each study.

D-serine Study 1

Male Fischer 344 rats were dosed with 5, 20, 50, 200 or 500 mg/kg D-serine in saline, whereas control animals were dosed with saline alone. Five animals were included in each dosing group with the exception of the 200 mg/kg group in which only four animals were included. Urine was collected in metabolic cages from animals prior to dosing and at 24, 48, 72 and 96 hours post-dose. One percent sodium azide, a bacteriostatic preservative, was added to each sample, and urine was frozen at -20°C within 1 hour of end point collection.

D-serine Study 2U (urine)

Male Fischer 344 rats were dosed with 200 or 500 mg/kg D-serine in saline, whereas control animals were dosed with saline alone. Five animals were included in each dosing group with the exception of the control group in which only four animals were included. Urine was collected from animals prior to dosing and at 12-hour intervals up to 168 hours post-dose. Protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) equal to 1/100 of the urine volume was added, and urine was frozen immediately at -20°C before being moved into a -80°C freezer within 1-7 days.

D-serine Study 2S (serum)

Male Fischer 344 rats were dosed with 200 or 500 mg/kg D-serine in saline, whereas control animals were dosed with saline alone. Ten animals were included in each dosing group. Five of the ten animals were sacrificed 12 hours post-dose. The remaining five animals were sacrificed 24 hours post-dose. Blood was collected from the inferior vena cava.

Evaluation of ALPCO rat RBP4 Immunoassay Kits

ELISA Kit Evaluation1

Enzyme-linked immunosorbent assays were performed using ALPCO Diagnostics dual mouse/rat RBP4 EIA kits (ALPCO Diagnostics, NH). For one urine sample expected to have measurable levels of RBP4, two 10-fold serial dilutions were performed, covering dilutions of 1:10 to 1:10,000 and dilutions 1:50 to 1:5,000. Additionally, the same 10-fold serial dilutions were performed for one urine sample expected to have baseline levels of RBP4.

ELISA Kit Evaluation 2

Four urine samples, two expected to have measurable levels of RBP4 and two expected to contain only baseline levels of the protein, were diluted 1:100, 1:500, 1:1000 and 1:5000. In addition, reference standard material at concentrations of 0, 0.375, 1.5 and 6 ng/mL of RBP4 were spiked into a baseline urine sample diluted 1:100, 1:1000 and 1:5000.

ELISA Kit Evaluation 3

Three urine sample expected to have measureable levels of RBP4 were diluted 1:10, 1:50 and 1:100. In addition, RBP4 was spiked into a baseline urine sample at 0 and 1.5 ng/mL. The urine was diluted 1:10, 1:50 and 1:100.

Urine and Serum Analysis using RBP4 ELISA

Enzyme-linked immunosorbent assays were performed as described in the ALPCO Diagnostics dual mouse/rat RBP4 EIA kit protocol. First, urine samples were diluted 1:10 and serum samples were diluted 1:1000 with diluent. One hundred microliters of diluted sample were added to the wells of 96-well microtiter plates coated with anti-mouse RBP4 polyclonal antibody and incubated for 1 hour at $37\,^{\circ}$ C. Plates were washed three times with wash solution. One hundred microliters of secondary anti-mouse RBP4 polyclonal antibody were added and incubated for 1 hour at $37\,^{\circ}$ C. Wells were washed three times, 100 uL of HRP-conjugated rabbit IgG was added to the wells, and plates were incubated for 1 hour at 37° C. Wells were washed five times, 100 uL of substrate solution was added, and plates were incubated 20 minutes at room temperature in the dark. Reactions were stopped by the addition of 100 uL of 1 M H_3PO_4. The absorbance (optical density [OD]) was measured at 450 nm with a microplate reader (Molecular Devices SpectraMax M2[e]).

Statistical Analysis

All data were the average of duplicate determinations. The results were expressed as mean values ± standard deviations (SD). Statistical methodology included analysis of variance (H0: $\mu_1 = \mu_2 = \ldots = \mu_k$ versus H_a: $\mu_i = \mu_j$). When a significant difference was found at $\alpha = .05$, Tukey's multiple comparisons were performed to identify which dosing groups differed significantly. To ensure validity of the analysis of variance, the Kolmogorov-Smirnov test of normality and Bartlett's test for equal variances were performed on the residuals. Where normality was not justified, the Kruskal-Wallis test was performed in lieu of the analysis of variance, followed by Tukey's multiple comparisons as appropriate. When the assumption of equal variances failed, transformations were performed to stabilize the variance.

2.4.4 RBP4 Results and Discussions

Evaluation of ALPCO RBP4 ELISA for use with rat urine samples

The ALPCO Diagnostics dual mouse/rat RBP 4 EIA kit is validated for use with rat serum and cell culture supernatants but not urine. Therefore, the kit was evaluated by three independent analyses in order to assess the kit's capability of detecting RBP4 in rat urine. First, the kit's capability of detecting RBP4 from in-house rat urine samples expected to have measurable levels of the protein was assessed. Second, the linearity of the results obtain from multiple dilutions of a urine sample was assessed. Third, the recovery efficiency of RBP4 from the urine matrix was assessed through a spike and recovery experiment.

ELISA Kit Evaluation 1

The kit insert recommend a starting dilution of 1:10,000 for serum samples. The first evaluation was designed to assay urine samples expected to have either baseline levels or relatively high levels of RBP4 at multiple dilutions to establish the appropriate sample dilution. Dilutions were chosen to include the manufacturer's recommended dilution factor for serum and to assess how low urine samples could be diluted without losing dilutional linearity. It should be noted that the result of the QC fell outside the acceptable range established by the manufacturer, being approximately half of what was expected (data not shown). The reference standard curve generated from the OD values did not have its points well distributed, being bottom heavy (Figure 25). Additionally the OD range for the reference standard was narrower than that indicated on the certificate of analysis provided by the manufacturer.   For the baseline urine sample, all ODs were below the range of the standard (Figure 25), thus below the limit of detection (data not shown). The other sample, expected to have relatively high levels of RBP4, had ODs within the range of the standard for dilution points 1:1000 and 1:5000 (Table 12). The remaining points were out of the standard range (high end for all except 1:10000 which was out of range at the low end). Further, the intra-assay CV for the results of the 1:1000 and 1:5000 points was 33.5%.

Table 12: RBP4 analysis of test animal urine at dilution factors of 10, 100, 1000, 10000, 50, 500 and 5000

* Outside of the range of the standard.

OD Value	Result (ng/mL)	Mean Result (ng/mL)	Std Deviation	%CV	Dilution Factor	Adjusted Result (ng/mL)
0.869	11.079					
*1.032	NA		NA		10	NA
*1.095	NA					
*1.052	NA		NA		100	NA
0.428	3.775					
0.548	5.371	4.573	1.129	24.7	1000	4573.351
*-0.082	NA					
*-0.076	NA		NA		1000	NA
*1.299	NA					
*1.373	NA		NA		50	NA
*0.943	NA					
*0.982	NA		NA		500	NA
0.111	1.008					
0.176	1.407	1.207	0.283	23.4	5000	6036.679
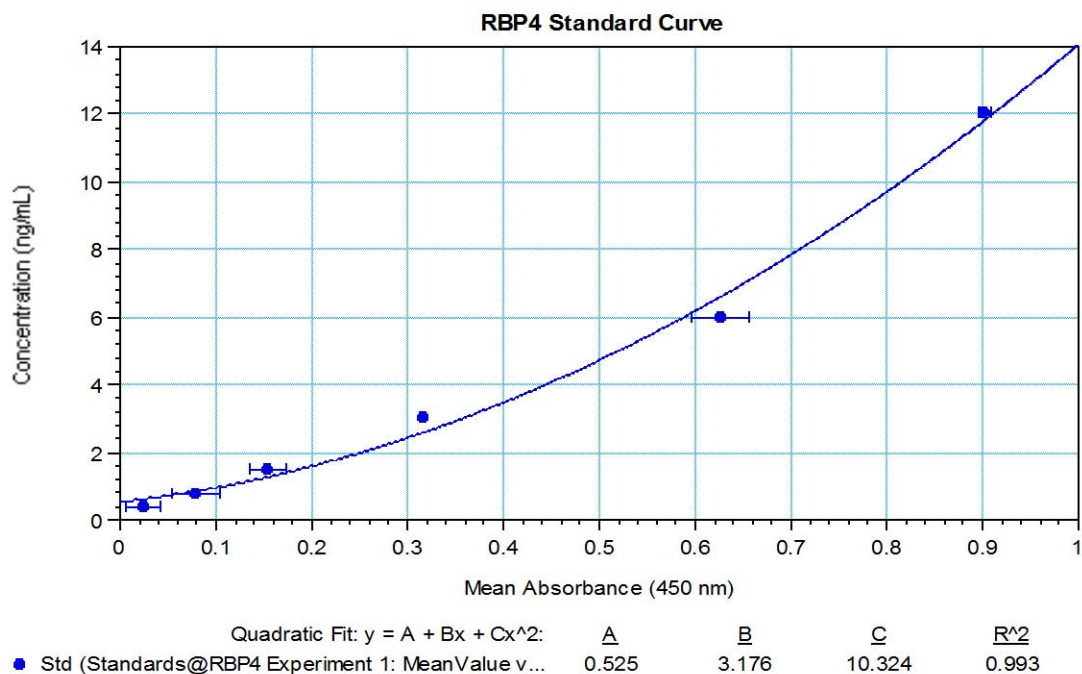
Figure 25: **RBP4 Standard Curve**

The ODs of the sample expected to have relatively high levels of RBP4 suggested the protein can be detected in urine. The dilution experiment indicated that, contrary to the manufacturer's recommendation for serum samples, a 1:10,000 dilution of urine samples is not required. Instead, RBP4 was measureable in urine at dilutions of 1:1000 to 1:5000. However, the failure of the QC sample was a concern. Therefore, a second assay was performed in order to repeat the dilution experiment at dilutions of 1: 100, 1:500, 1:1000 and 1:5000. In addition, the evaluation included a spike and recovery experiment in which 0, 0.375, 1.5 and 6 ng/mL RBP4 (reference standard) were spiked into urine diluted 1:100, 1:1000 and 1:5000

ELISA Kit Evaluation 2

While the same lot of kit was used for evaluation 2, a new kit with previously unopened reference standard and QC was used. As with the first experiment, the QC result fell outside the acceptable range established by the manufacturer. Again, the OD values generated from the reference standards were narrower than those listed on the certificate of analysis included with the kit. At dilutions of 1: 100, 1:500, 1:1000 and 1:5000, RBP4 was not detected in the urine from two animals expected to contain baseline levels of the protein (data not shown). However, relatively high levels of RBP4 were detected in urine from two animals expected to contain measurable levels of the protein (Table 13). Minimum dilutions of 1:500 were required for ODs to fall within the range of the standard curve. The dilutional linearity for points 1:500 to 1:5000 was adequate, with intra-assay CVs of 21.5 and 24.5%.

Table 13: RBP4 dilutional linearity between urine samples diluted 1:100, 1:500, 1:1000 and 1:5000
Outside of the range of the standard

Sample	Dilution Factor	Mean OD	Result (pg/mL)	Duplicate %CV	Adjusted Result (μg/mL)	Mean	%CV
1	100	Out of Range	NA			3627.763	21.5
	500	1.212	6.218	6.2	3108.7		
	1000	1.013	4.523	7.8	4522.5		
	5000	0.291	0.650	34.6	3251.9		
2	100	Out of Range	NA			2907.484	24.5
	500	1.273	6.917	5.9	3458.4		
	1000	0.800	3.160	17.4	3160.1		
	5000	0.236	0.421	32.6	2103.8		

For the spike and recovery experiment, 0, 0.375, 1.5, and 6 ng/mL RBP4 (reference standard) were spiked into 1:100, 1:1000, and 1:5000 dilutions of a baseline urine sample (Table 14). All ODs for the 0 and 0.375 ng/mL spikes were out of range of the standard curve. For the 1.5 and 6 ng/mL spikes the recovery was generally good, ranging from 50-90% for each point. Further, the urine dilutional linearity was good for the spiked concentration of 6 ng/mL, with a CV between urine dilutions of only 5%.

Table 14: Spike and recovery of 1.5 and 6 ng/mL RBP4

RBP4 (reference standard material) was aliquoted in 1:100, 1:1000 and 1:5000 dilutions of a baseline urine sample

Spiked Conc. (ng/mL)	Dilution Factor	Mean Adj. Result (ng/mL)	Replicate %CV	Percent Recovery	Mean Dilution Result (ng/mL)	Mean %CV	Mean Percent Recovery
1.5	100	1.352	5.5	90.1			
1.5	1000	0.755	15.0	50.3	1.117	28.47	74.4
1.5	5000	1.243	12.5	82.9			
6	100	4.362	1.8	72.7			
6	1000	4.787	0.9	79.8	4.662	5.59	77.7
6	5000	4.836	2.9	80.6			

Despite the QC sample provided with the kit falling outside the range specified by the manufacturer, rat urine samples from animals expected to contain measureable levels of RPB4 (i.e. samples from rats dosed with 200 or 500 mg/kg of D-serine) produced strong RBP4 signals with this kit. Based on evaluations 1 and 2, the kit is suitable for the analysis of rat urine. A third dilution experiment was performed in order to assess whether urine samples can be diluted as low as 1:10. In addition, a spike and recovery experiment was performed. RBP4 was spiked into a baseline urine sample at 0 and 1.5 ng/mL. The urine was diluted 1:10, 1:50 and 1:100.

ELISA Kit Evaluation 3

The analysis of urine at dilution factors of 10, 50 and 100 suggests urine samples can be diluted as low as 1:10 (Table 15). While the intra-assay CV between dilution points 1:10, 1:50 and 1:100 was greater than 20% for sample 3, the dilutional linearity was acceptable for samples 1 and 2, with CVs between urine dilutions of 16.2 and 14.9%, respectively. In addition, dilutional linearity was observed during the spike and recovery experiment. The CV between urine dilutions of samples spiked with 1.5 ng/mL of RBP4 was 1.4% (Table 16). The recoveries of RBP4 from a baseline urine sample spiked with 1.5 ng/mL and diluted 1:10, 1:50 and 1:100 were consistent at 84.5%, 85.5% and 87%, respectively.

Based on all three kit evaluations, the lowest recommended starting dilution of urine to be assayed with the ALPCO Diagnostics dual mouse/rat RBP4 EIA kit is 1:10.   Neither ALPCO Diagnostics nor their manufacturing partner was able to aid AFRL in determining the cause for the QC values falling outside the acceptable range. Because the QC values obtained in this laboratory were very consistent (CV < 15%) between assays, even of various lots, ALPCO Diagnostic recommended establishing an internal QC range and deemed the results obtained with the assays acceptable.

Table 15: **RBP4 Dilutional linearity between urine samples diluted 1:10, 1:50 and 1:100**

Sample	Dilution Factor	Mean OD	Result (pg/mL)	Duplicate %CV	Adjusted Result (µg/mL)	Mean	%CV
1	10	0.357	1.876	6.3	18.759	21.306	16.2
	50	0.104	0.505	14.2	25.231		
	100	0.037	0.199	14.4	19.928		
2	10	0.360	1.896	9.6	18.961	21.192	14.9
	50	0.096	0.468	4.2	23.422		
	100	Out of Range	NA				
3	10	1.150	9.775	13.2	97.752	139.578	33.0
	50	0.625	3.776	0.5	188.817		
	100	0.262	1.322	2.8	132.164		

Table 16: Spike and recovery of 1.5 ng/mL RBP4
RBP4 (reference standard material) was aliquoted in a 1:10, 1:50 and 1:100 dilution of baseline urine sample

Spiked Conc. (ng/mL)	Dilution Factor	Mean Result (ng/mL)	Replicate %CV	Percent Recovery	Mean Dilution Result (ng/mL)	Mean %CV	Mean Percent Recovery
1.5	10	1.267	4.5	84.5	1.284	1.4	85.6
1.5	50	1.282	7.7	85.5			
1.5	100	1.304	4.4	87.0			

RBP4 expression profiling in rat urine samples from D-serine studies

<u>D-Serine Study 1</u>

The known nephrotoxin D-serine was used to induce sub-clinical kidney damage at the proximal straight tubules in order to evaluate whether RBP4 could serve as an early predictor of nephrotoxicity. Doses of 20, 50, 200 or 500 mg/kg D-serine were administered intraperitoneally in 0.9% saline. Control animals were dosed with saline alone. Urine samples were collected at 24 hours intervals in a 96 hour period. RBP4 was quantitated by sandwich ELISA and the biomarker expression profile was determined.

Significant biomarker profile changes in RBP4 were seen in urine only in the 200 and 500 mg/kg D-serine dose groups. No significant profile changes were seen at the 5, 20 or 50 mg/kg D-serine dose groups (data not shown). Kidney histopathology data and clinical chemistry data (TBIL, CREA, BUN, and Total Protein) confirmed the initiation of kidney damage around the 200 mg/kg dose (data not shown). The urine profile of the biomarker peaked at 24 hours, reaching approximately 5400 ng/mL in dosing groups 200 and 500 mg/kg (Figure

26).   At peak expression, dosing groups 200 and 500 mg/kg were significantly larger than the control group. However, the 200 and 500 mg/kg dosing groups were not significantly different from each other ($p < 0.05$). At the 72 hour time-point, RBP4 levels decreased to as low as 541 ng/mL and 513 ng/mL in dosing groups 200 and 500 mg/kg, respectively. At the 96 hour time-point, RBP4 levels decreased to 195 ng/mL in dosing group 200 mg/kg. However, in dosing group 500 mg/kg, the RBP4 concentration remained at 512 ng/mL, indicating a plateau in the excretion of the protein.
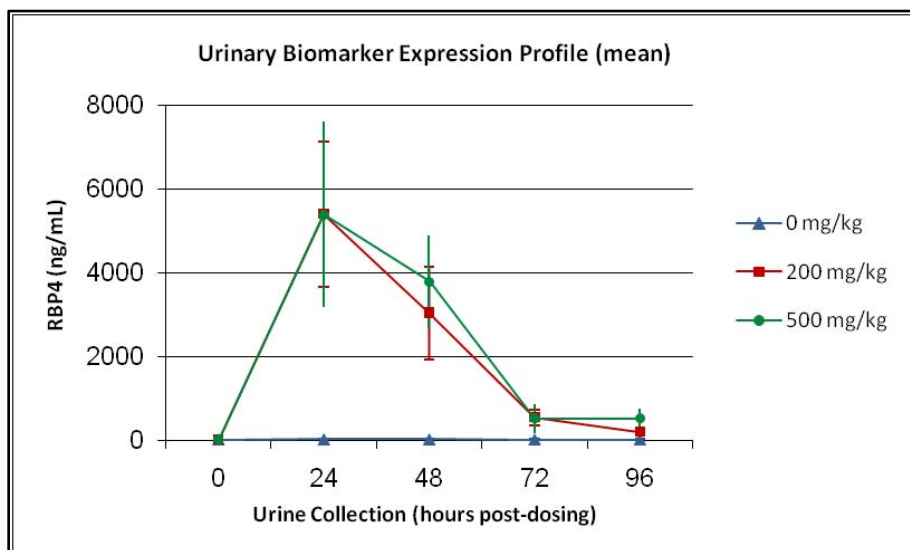
Figure 26: **Measurement of urinary RBP4 by ELISA versus time after D-serine administration**

D-Serine Study 2U

A second time-course experiment was conducted to confirm that maximal expression of urinary RBP4 occurs at 24 hours, not 12 or 36 hours. Again, D-serine was used to induce sub-clinical kidney damage at the proximal straight tubules. Doses of 200 or 500 mg/kg D-serine were administered intraperitoneally in 0.9% saline. Control animals were dosed with saline alone. Urine samples were collected at 12 hours intervals in a 168 hour period. RBP4 was quantitated by sandwich ELISA and the biomarker expression profile was determined.

As previously observed in the original time-course experiment, significant biomarker profile changes in RBP4 were seen in urine in the 200 and 500 mg/kg D-serine dose groups. Kidney histopathology data and clinical chemistry data (TBIL, CREA, BUN, and Total Protein) confirmed sub-clinical kidney damage at the 200 and 500 mg/kg doses (data not shown). The urine profile of the biomarker peaked at 24 hours, reaching 12,289 ng/mL and 12,793 ng/mL in dosing groups 200 and 500 mg/kg, respectively (Figure 27).

49
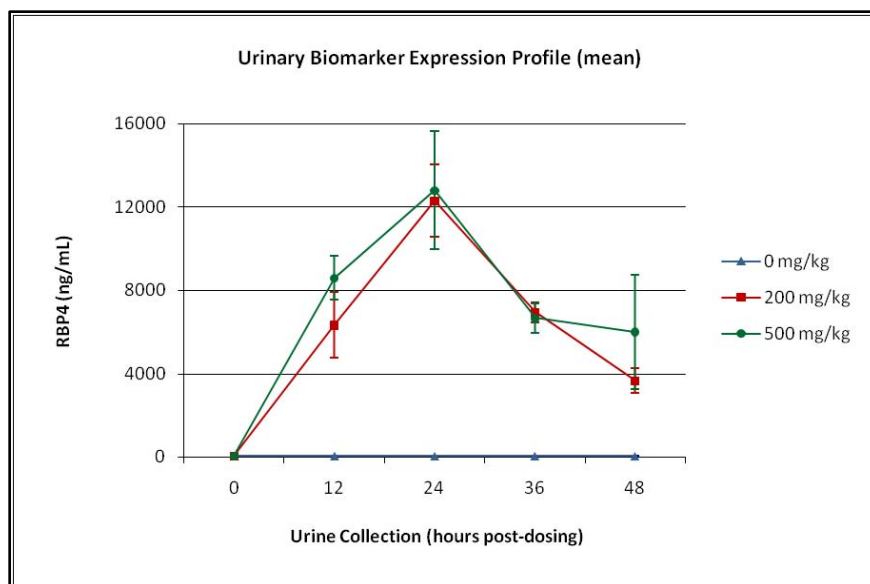
Figure 27: Measurement of urinary RBP4 by ELISA versus time after D-serine administration (Study 2U)

At peak expression, dosing groups 200 and 500 mg/kg were significantly higher than the control group. However, the 200 and 500 mg/kg dosing groups were not significantly different from each other ($p < 0.05$). At the 36 hour time-point, RBP4 levels decreased to 6,925 ng/mL and 6,647 ng/mL in dosing groups 200 and 500 mg/kg, respectively. RPB4 protein levels continued to decrease to as low as 3,659 ng/mL and 6,001 ng/mL in dosing groups 200 and 500 mg/kg, respectively, at the 48 hour time-point. As discussed previously (Section 2.1), the profile data was normalized and graphed using either urine volume-weighted (Figure 28), creatinine-adjusted (Figure 29), or BACC baseline adjustments (Figure 30) for comparisons to RBP4 published data.
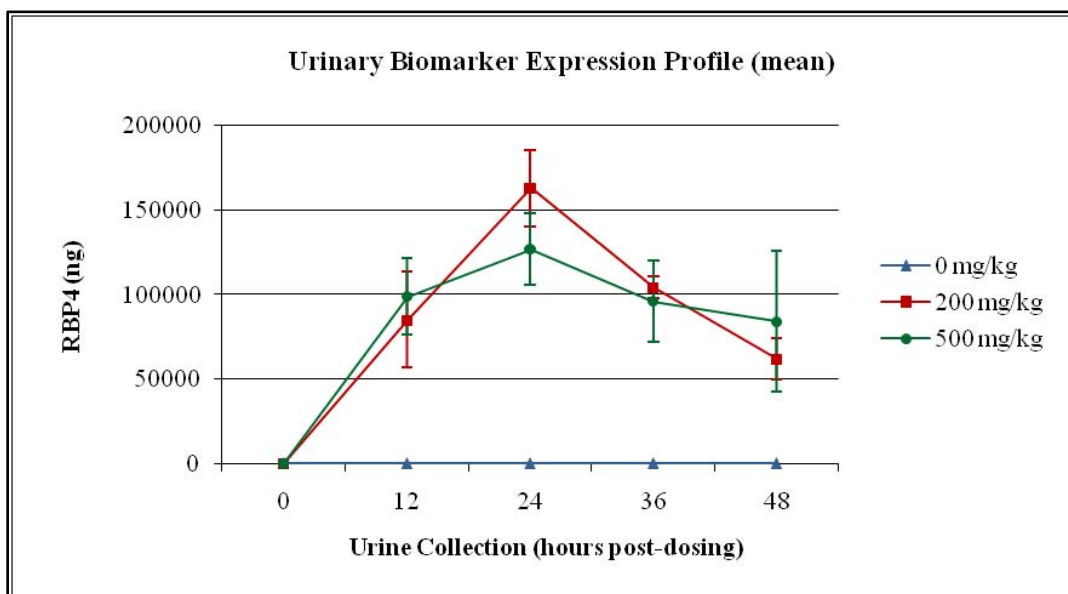
Figure 28: Measurement of urinary RBP4 (urine volume-weighted) versus time after D-serine administration (Study 2U)
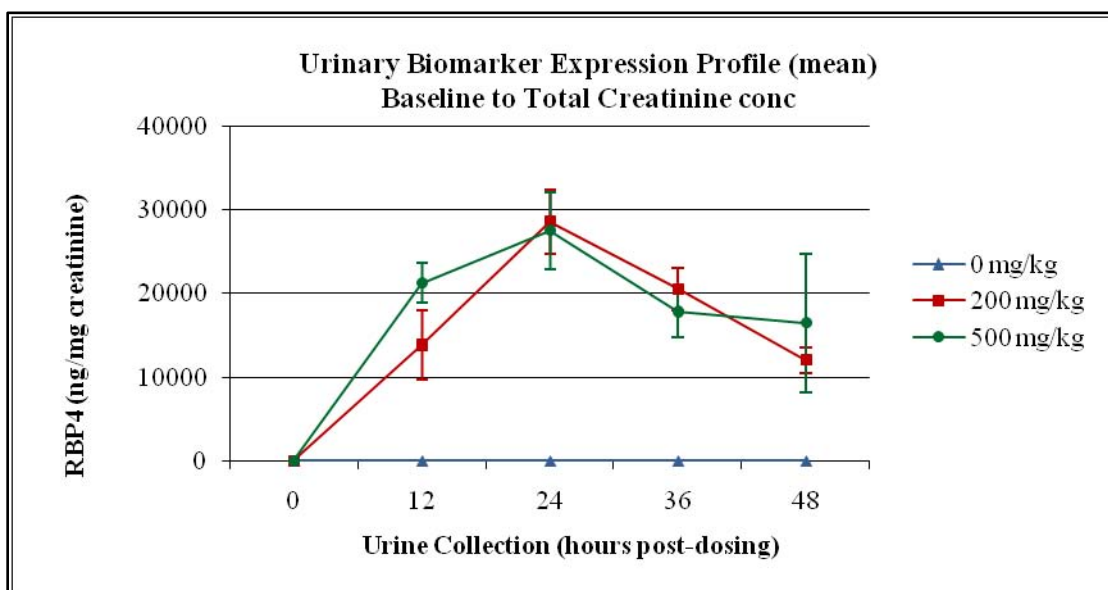
Figure 29: **Measurement of urinary RBP4 (ng/mg creatinine) versus time point after D-serine administration (Study 2U)**
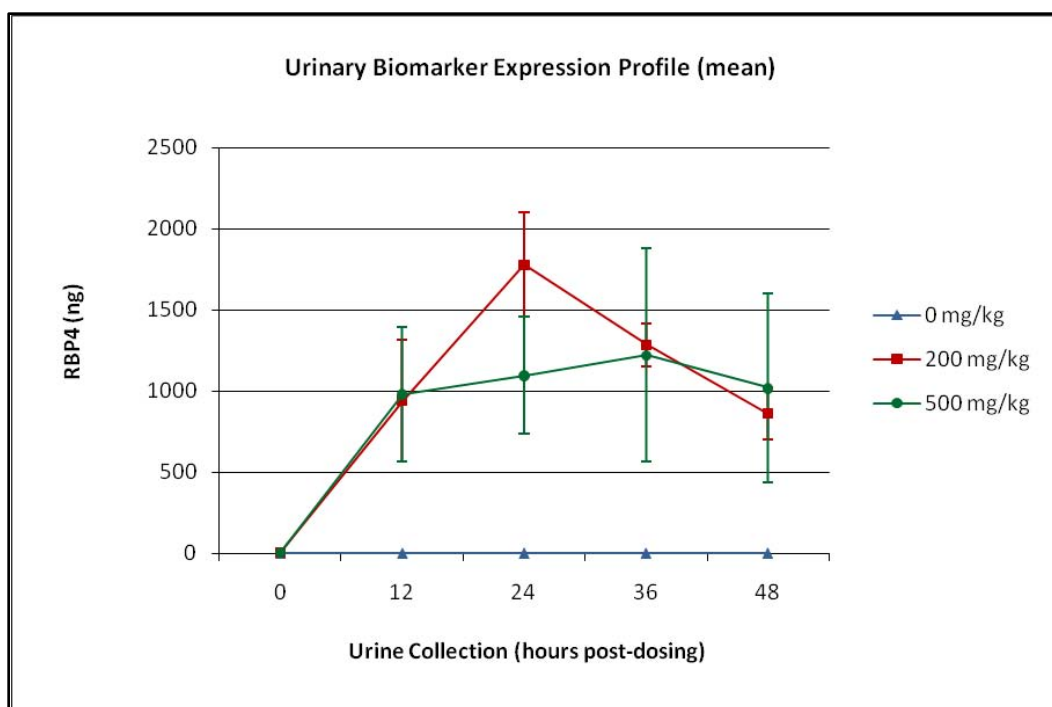
Figure 30: Measurement of urinary RBP4 (BACC adjusted) versus time point after D-serine administration (Study 2U)

RBP4 expression profiling in rat serum samples from D-serine studies

D-Serine Study 2S

In order to determine whether changes in the RBP4 expression signal increases can be detected in blood earlier than in urine, the putative biomarker was quantitated in serum samples using ELISA. Male Fischer 344 rats were dosed with 200 or 500 mg/kg D-serine in saline, whereas control animals were dosed with saline alone. Ten animals were included in each dosing group. Five of the ten animals were sacrificed 12 hours post-dosing. The remaining five animals were sacrificed 24 hours post-dosing.

A two-way analysis of variance (ANOVA) demonstrated that RBP4 expression did not differ significantly between the 12 and 24 hour time points of dosing groups 0, 200 and 500 mg/kg. In the control group, the biomarker reached 64,317 ng/mL and 70,569 ng/mL at the 12 and 24 hour time points, respectively (Figure 31). In the 200 mg/kg dosing group, RBP4 reached 45,851 ng/mL and 38,365 ng/mL at the 12 and 24 hour time points, respectively. In the 500 mg/kg dosing group, RBP4 reached 33,939 ng/mL and 31,640 ng/mL at the 12 and 24 hour time points, respectively. A one-way ANOVA between dosing groups on pooled 12 and 24 hour data showed no significant differences in the expression of RBP4 between the 200 mg/kg and 500 mg/kg dosing groups, but showed that the control group was significantly higher than both dosing groups. Dilutions were adjusted accordingly to get samples to fall within the range of the assay.

Figure 31: Measurement of serum RBP4 by ELISA at 12 h and 24 h after D-serine administration.

2.5 Heme Oxygenase 1 (HO-1)

2.5.1 HO-1 Summary

An examination of current literature identified heme oxygenase-1 (HO-1) as a potential biomarker predictive of acute, chronic, chemically-induced, or disease-induced kidney damage. The protein expression profile of HO-1 in urine was examined in a time/dose experiment using several concentrations of the known nephrotoxin D-serine. D-serine has been shown to specifically induce kidney damage at the proximal straight tubules. Male Fischer 344 rats were dosed with 5, 20, 50, 200, or 500 mg/kg D-serine in saline, whereas control animals were dosed with saline alone. D-serine doses were administered intraperitoneally in 0.9% saline, while control animals were dosed with saline alone. Urine was collected from animals prior to dosing and at 24, 48, 72 and 96 hours post-dose and was frozen within one hour of end point collection. The urine samples were assayed for HO-1 levels using commercially available ELISA. Biomarker levels remained constant for animals dosed with 0, 5, 20, and 50 mg/kg D-serine throughout the time course. However, for animals dosed with 200 and 500 mg/kg D-serine, significant increases were observed with levels peaking at 24 hours post-dose. HO-1 levels dropped to pre-dose levels at 72 hours post-dosing. A second time-course experiment was conducted in order to confirm the peak response times of the biomarker. In Study 2U, samples were collected at a more frequent 12 hour interval for a longer 168 hour period. Using a narrower sample collection period, significant increases in HO-1 were observed at 12 hours post-dose with a return to baseline at 48 hours post-dose. In addition to assaying urine samples, serum samples were assayed by ELISA for HO-1. Blood was collected at 12 and 24 hours post-dose in animals injected with 0, 200, and 500 mg/kg of D-serine. No statistically significant difference in HO-1 levels were observed between the 12 and 24 hour time points of dosing groups 0, 200 and 500 mg/kg. Multiple statistical analyses also did not demonstrate significant differences between dosing groups at the two time points. Along with the existing journal based data, these expression profiles in response to a kidney toxin indicate that the detection of urinary HO-1 is a potential noninvasive strategy to predict the early onset of renal damage. Unlike urinary HO-1 levels, serum HO-1 concentrations do not change to significant levels in response to nephrotoxin exposures. In light of these data, only HO-1 levels monitored in the urine can be considered an appropriate biomarker indicator to subclinical renal damage.

2.5.2 HO-1 Introduction

Heme Oxygenase exists as three isoforms, only one of which (HO-1) is induced in almost all cells in response to stressors such as infection, toxins, mechanical damage (excellent review in Morse et al., 2005). The gene regulation of HO-1 is fairly complex, consisting of a stress response element present in two upstream enhancer regions which has been shown to be used in response to all HO-1 induction examined (Alam et al., 2004).

HO-1 is the rate-limiting enzyme in heme degradation which cleaves the cellular heme group to form biliverdin, carbon monoxide, and ferritin (Kikuchi 2004). Further studies have indicated that HO-1, once activated, provides the cell with capabilities of protecting against inflammation and apoptosis, as well as provide defenses against sepsis and atherosclerosis (Stocker, 1990; Yachie et al., 1999; Otterbein et al., 2000). In the kidney, basal levels of HO-1 are fairly low, but upon induction are seen at relatively high levels primarily in the proximal

tubules. Therefore, monitoring of HO-1 levels in urine may be effective in detecting low level damage to the kidney. The use of HO-1 as a marker of oxidative injury has been examined in multiple disease models in the kidney (reviewed in Jarmi et al., 2009) such as atherosclerosis, transplant rejection, hypertension and AKI (Hill-Kapturczak et al., 2002). Studies using the nephrotoxin cyclosporine to initiate tubulointerstitial fibrosis (Rezzani et al., 2005) as well as cisplatin induced damage (Shiraishi et al., 2000) demonstrated that HO-1 was induced in the kidney and provided some protection against the effects of the toxin. Using unilateral ureteral obstruction to mimic renal interstitial fibrosis in the mouse, HO-1 protein and mRNA was shown to be upregulated in the kidney as early as 12 hours (Kawada et al., 1999). In light of the many studies examining HO-1 induction in kidney injury or disease models, it is surprising that there are limited data on HO-1 levels in urine or serum/plasma in response to renal injury or dysfunction.

2.5.3 HO-1 Methods, Assumptions, and Procedures

Urine and Serum Collection

More detailed descriptions of the animal studies are described in *Biomarkers of Exposure to Toxic Substances, Vol 1* (DelRaso et al., Sep 2009). However, for clarity of this section, we include brief descriptions of each study.

D-serine Study 1

     Male Fischer 344 rats were dosed with 5, 20, 50, 200 or 500 mg/kg D-serine in saline, whereas control animals were dosed with saline alone. Five animals were included in each dosing group with the exception of the 200 mg/kg group in which only four animals were included. Urine was collected in metabolic cages from animals prior to dosing and at 24, 48, 72 and 96 hours post-dosing. One percent sodium azide, a bacteriostatic preservative, was added to each sample, and urine was frozen at -20°C within 1 hour of end point collection.

D-Serine Study 2U

     Male Fischer 344 rats were dosed with 200 or 500 mg/kg D-serine in saline, whereas control animals were dosed with saline alone. Five animals were included in each dosing group with the exception of the control group in which only four animals were included. Urine was collected from animals prior to dosing and at 12-hour intervals up to 168 hours post-dosing. Protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) equal to 1/100 of the urine volume was added, and urine was frozen immediately at -20°C before being moved into a -80°C freezer within 1-7 days.

D-Serine Study 2S

     Male Fischer 344 rats were dosed with 200 or 500 mg/kg D-serine in saline, whereas control animals were dosed with saline alone. Ten animals were included in each dosing group. Five of the ten animals were sacrificed 12 hours post-dosing. The remaining five animals were sacrificed 24 hours post-dosing. Blood was collected from the inferior vena cava.

HO-1 Immunoassay Evaluation in rat urine samples.

Kit Evaluation 1

Enzyme-linked immunosorbent assays were performed using Takara rat heme oxygenase-1 EIA kits (Takara Bio Inc., Japan). Eight urine samples expected to contain measurable levels of HO-1 were diluted 1:4 and 1:12.

Kit Evaluation 2

Five urine samples, expected to contain measurable levels of HO-1, were diluted 1:2 and 1:4. In addition, reference standard material at concentrations of 0, 0.25 and 1 ng/mL of HO-1 was spiked into a baseline urine sample diluted 1:2 and 1:4.

HO-1 Profiling in urine and serum samples from D-serine dosed rats.

Enzyme-linked immunosorbent assays were performed as described in the Takara Bio rat heme oxygenase-1 EIA kit protocol. First, urine and serum samples were diluted 1:2 with sample diluent. One hundred microliters of diluted sample were added to the wells of 96-well microtiter plates coated with murine monoclonal antibody to rat heme oxygenase-1 and incubated for 1 hour at 20-30° C. Plates were washed three times with wash solution (phosphate-buffered saline containing 0.1% Tween 20). One hundred microliters of antibody-POD conjugate (horseradish peroxidase conjugated murine monoclonal antibody to rat heme oxygenase-1) were added and incubated for 1 hour at 20-30° C. Wells were washed four times, 100 uL of substrate solution (hydrogen peroxide and tetramethylbenzidine in a buffered solution) was added to the wells, and plates were incubated for 15 minutes at 20-30° C. Reactions were stopped by the addition of 100 uL of 1 N H_2SO_4. The absorbance (optical density [OD]) was measured at 450 nm with a microplate reader (Molecular Devices SpectraMax M2^e).

Statistical Analysis

All data were the average of duplicate determinations. The results were expressed as mean values ± standard deviations (SD). Statistical methodology included analysis of variance (H0: $\mu_1 = \mu_2 = \ldots = \mu_k$ versus H_a: $\mu_i = \mu_j$). When a significant difference was found at $\alpha = .05$, Tukey's multiple comparisons were performed to identify which dosing groups differed significantly. To ensure validity of the analysis of variance, the Kolmogorov-Smirnov test of normality and Bartlett's test for equal variances were performed on the residuals. Where normality was not justified, the Kruskal-Wallis test was performed in lieu of the analysis of variance, followed by Tukey's multiple comparisons as appropriate. When the assumption of equal variances failed, transformations were performed to stabilize the variance.

2.5.4 HO-1 Results and Discussions

Takara HO-1 ELISA Kit Evaluation in rat urine

The Takara Bio rat heme oxygenase-1 EIA kit is validated for use with rat blood and organs but not urine. Therefore, the kit was evaluated by two independent analyses in order to assess the kit's capability of detecting HO-1 in rat urine. First, the kit's capability of detecting HO-1 from in-house rat urine samples expected to have measurable levels of the protein was assessed. In addition, the linearity of the results obtained from multiple dilutions of a urine sample was evaluated. Second, the recovery efficiency of HO-1 from the urine matrix was assessed through a spike and recovery experiment.

ELISA Kit Evaluation 1

The kit insert recommends starting dilutions from 1:2 to 1:8 for serum or organ extract samples. The first evaluation was designed to assay urine samples expected to have measurable levels of HO-1 at two dilutions to establish the appropriate sample dilution. Dilutions were chosen to include the manufacturer's recommended dilution factor for serum and to assess how low urine samples could be diluted without losing dilutional linearity.

The ODs of six out of the eight samples fell below the range of the standard curve (Figure 32). However, for the two samples with quantifiable levels of HO-1, both dilution points were within the range of the standard curve and the inter-dilutional CVs were < 30% (Table 17). Dilutions as high as 1:12 were not necessary for this selection of samples, and all samples had ODs low enough that assaying samples at lower dilutions would be desirable provided dilutional linearity is retained.

HO-1 Standard Curve

4-P Fit: y = (A - D)/(1 + (x/C)^B) + D:	A	B	C	D	R^2
● Std (Standards@HO-1 Evaluation 1: Conc vs Mean...	0.056	1.229	23.819	12.539	1

Curve Fit Option - Fixed Weight Value: 1
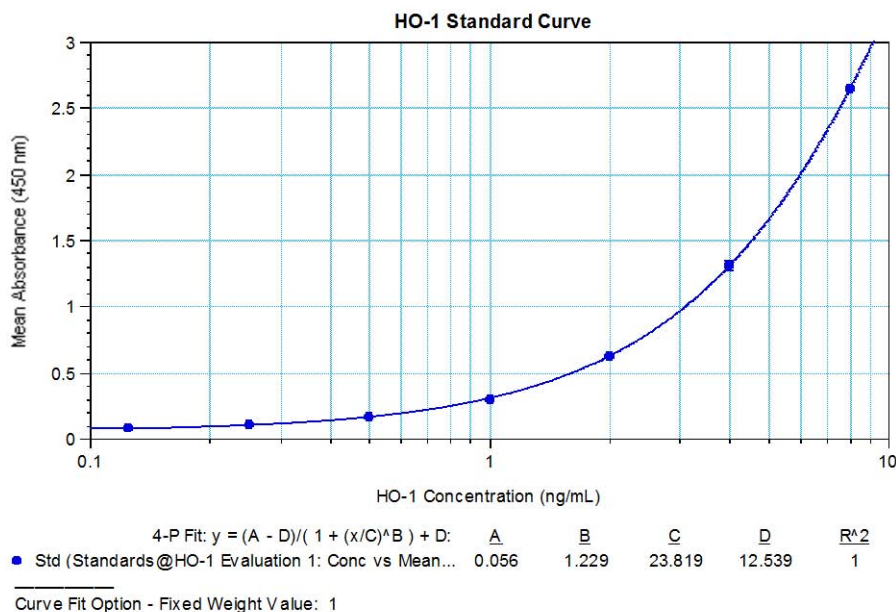
Figure 32: **HO-1 Standard Curve**

Table 17: **HO-1 analysis of test animal urine at dilution factors of 4 and 12**
* ODs below the lowest non-zero point of the standard curve.

Sample	Dilution Factor	Mean OD (Intra)	Intra-dilutional Mean Result (ng/mL)	Std. Dev. (Intra)	CV% (Intra)	Adjusted Result (ng/mL)	Inter-dilutional Mean Result (ng/mL)	Std. Dev. (Inter)	CV% (Inter)
1	4	0.174	0.539	0.030	5.6	2.157	2.691	0.754	28.04%
	12	0.107	0.269	0.022	8.3	3.224			
2	4	*0.059	NA						
	12	*0.058							
3	4	*0.069	NA						
	12	*0.056							
4	4	*0.054	NA						
	12	*0.055							
5	4	0.266	0.868	0.001	0.1	3.472	3.735	0.371	9.94%
	12	0.122	0.333	0.005	1.4	3.997			
6	4	*0.064	NA						
	12	*0.061							
7	4	*0.051	NA						
	12	*0.050							
8	4	*0.058	NA						
	12	*0.055							

The dilution experiment indicated that dilutions of 1:4 or less would be appropriate for analyzing urine samples. A second experiment was performed in order to determine whether a dilution of less than 1:4 would have adverse effects on quantitation, such as interference or background signal. Five samples expected to contain measurable quantities of HO-1 were diluted at 1:2 and 1:4. In addition, the evaluation included a spike and recovery experiment in which 1:2 and 1:4 dilutions of baseline urine expected to have no measurable HO-1 were spiked with concentrations of HO-1 that represented the middle and low regions of the standard range.

<u>ELISA Kit Evaluation 2</u>

At a dilution of 1:2, all five of the urine samples expected to contain measurable levels of HO-1 fell within the quantifiable range of the standard curve. At a dilution of 1:4, four out of the five samples fell within the range of the standard curve. The dilutional linearity was acceptable for samples 1 through 4, with CVs ranging from 1.5 to 8.3% between urine dilutions (Table 18).

Table 18: HO-1 dilutional linearity between urine samples diluted 1:2 and 1:4

* ODs below the lowest non-zero point of the standard curve.

Sample	Dilution Factor	Mean OD	Result (ng/mL)	Duplicate %CV	Adjusted Result (ng/mL)	Mean	%CV
1	2	0.407	1.228	1.1	2.455	2.596	7.658
	4	0.233	0.684	3.3	2.736		
2	2	0.127	0.314	6.4	0.629	0.668	8.34
	4	0.093	0.177	7.9	0.707		
3	2	0.541	1.626	2.8	3.251	3.326	3.19
	4	0.284	0.850	0.6	3.401		
4	2	0.564	1.692	2.5	3.385	3.420	1.45
	4	0.289	0.864	4.1	3.455		
5	2	0.101	0.211	25.4	0.421	NA	NA
	4	*0.076	NA				

For the spike and recovery experiment, 0, 0.25 and 1 ng/mL HO-1 (reference standard) were spiked into 1:2 and 1:4 dilutions of a baseline urine sample. These concentrations represented the middle and low regions of the standard curve. Not enough standard material was provided in the kit to perform a third spike at a higher concentration.

All ODs for the 0.25 and 1 ng/mL spikes were within the range of the standard curve. The recovery of HO-1 at the 1:2 dilution was 100.8 and 82.5% for the 0.25 and 1 ng/mL spikes, respectively (Table 19). At the 1:4 dilution, the recovery of HO-1 was 103.6 and 88.5% for the 0.25 and 1 ng/mL spikes, respectively. In addition to satisfactory recovery of the protein from the urine matrix, dilutional linearity was observed during the spike and recovery experiment. The CV between urine dilutions of samples spiked with 0.25 ng/mL of HO-1 was 1.9%. The CV between urine dilutions of samples spiked with 1 ng/mL of HO-1 was 5.0% (not shown).

Table 19: Spike and recovery of 0.25 and 1 ng/mL HO-1

*HO-1(reference standard material) was aliquoted into 1:2 and 1:4 dilutions of a baseline urine sample; * ODs below the lowest non-zero point of the standard curve.*

Spiked HO-1 Conc. (ng/mL)	Urine Dilution Factor	Replicate OD Values	Replicate Result (ng/mL)	Mean Result (ng/mL)	CV%	HO-1 Recovered (spike - ng/mL)	Percent Recovery
0	2	0.081	0.130	0.108	28.8	0	NA
		*0.072	0.086				
0.25		0.134	0.342	0.360	6.9	0.252	100.8
		0.144	0.377				
1		0.308	0.923	0.933	1.4	0.825	82.5
		0.313	0.942				
0	4	0.081	0.130	0.086	73.1	0	NA
		*0.063	0.041				
0.25		0.144	0.379	0.345	13.8	0.259	103.6
		0.126	0.312				
1		0.322	0.968	0.971	0.4	0.885	88.5
		0.324	0.973				

Based on evaluations 1 and 2, the kit is suitable for the analysis of rat urine. HO-1 was detected in rat urine samples from animals expected to contain measureable levels of the protein. The kit displayed > 80% recovery of HO-1 spiked into urine. The analyses indicated that samples can be diluted as low as 1:2 without compromising dilutional linearity. Therefore, the recommended starting dilution of urine to be assayed with the Takara Bio rat heme oxygenase-1 EIA kit is 1:2.

HO-1 Profiling in rat urine samples from D-serine dosed animals.

D-Serine Study 1

The known nephrotoxin D-serine was used to induce sub-clinical kidney damage at the proximal straight tubules in order to evaluate whether HO-1 could serve as an early predictor of nephrotoxicity. Doses of 20, 50, 200 or 500 mg/kg D-serine were administered intraperitoneally in 0.9% saline. Control animals were dosed with saline alone. Urine samples were collected at 24 hours intervals in a 96 hour period. HO-1 was quantitated by sandwich ELISA and the biomarker expression profile was determined.

Neither a limit of quantitation, nor a limit of detection was reported in the rat HO-1 EIA kit manual. For the purposes of this analysis, protein concentrations below that of the lowest calibrator (125 pg/mL) were considered below the lower limit of quantitation (LLOQ).

Concentrations of samples that fell below the LLOQ were, nevertheless, calculated in order to perform statistical analysis between dosing groups. Protein levels in the control group fell below the LLOQ at all time points.

Significant biomarker profile changes in HO-1 were seen in urine only in the 200 and 500 mg/kg D-serine dose groups. No significant profile changes were seen at the 5, 20 or 50 mg/kg D-serine dose groups (data not shown). Kidney histopathology data and clinical chemistry data (TBIL, CREA, BUN, and Total Protein) confirmed the initiation of kidney damage around the 200 mg/kg dose (data not shown). The urine profile of the biomarker peaked at 24 hours, reaching approximately 2,496 pg/mL and 3,322 pg/mL in dosing groups 200 and 500 mg/kg, respectively (Figure 33). At peak expression, dosing groups 200 and 500 mg/kg were significantly larger than the control group. However, the 200 and 500 mg/kg dosing groups were not significantly different from each other ($p<0.05$). At the 48 hour time-point, HO-1 levels decreased to as low as 364 pg/mL and 214 pg/mL in dosing groups 200 and 500 mg/kg, respectively.
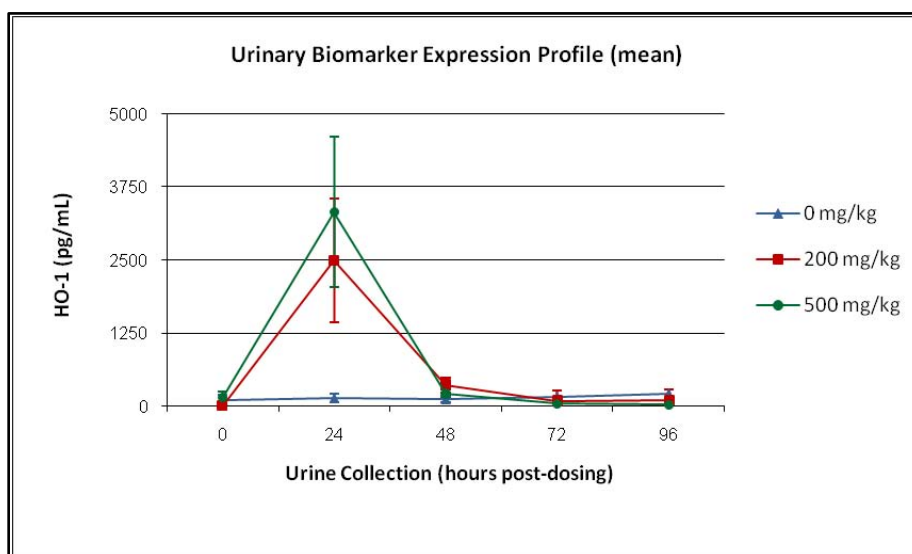
Figure 33: **Measurement of urinary HO-1 by ELISA versus time after D-serine administration**

In both dosing groups, HO-1 levels continued to decrease below the LLOQ at the 72 and the 96 hour time-points, indicating a plateau in the excretion of the protein.

D-Serine Study 2U

A second time-course experiment was conducted in order to confirm that HO-1 peaks at 24 hours, not 12 or 36 hours. Again, D-serine was used to induce sub-clinical kidney damage at the proximal straight tubules. Doses of 200 or 500 mg/kg D-serine were administered intraperitoneally in 0.9% saline. Control animals were dosed with saline alone. Urine samples

were collected at 12 hours intervals in a 168 hour period. HO-1 was quantitated by sandwich ELISA and the biomarker expression profile was determined.

As previously observed in the original time-course experiment, significant biomarker profile changes in HO-1 were seen in urine in the 200 and 500 mg/kg D-serine dose groups. Kidney histopathology data and clinical chemistry data (TBIL, CREA, BUN, and Total Protein) confirmed sub-clinical kidney damage at the 200 and 500 mg/kg doses (data not shown). Contrary to the previously observed 24 hour peak, the second time course experiment demonstrated the urine profile of the biomarker in fact peaks at 12 hours, reaching 5,428 pg/mL and 8,588 pg/mL in dosing groups 200 and 500 mg/kg, respectively (Figure 34).
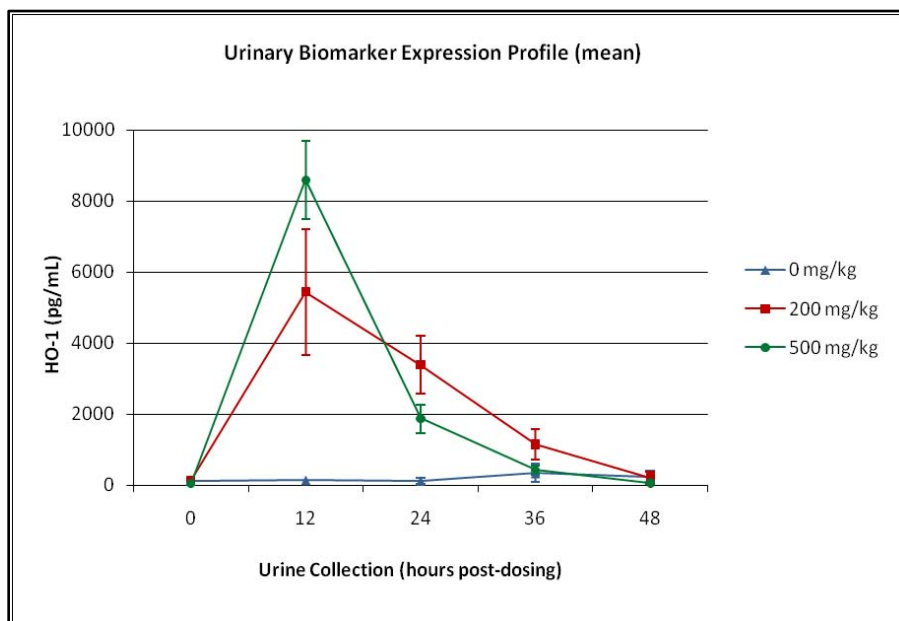
Figure 34: Measurement of urinary HO-1 by ELISA versus time after D-serine administration

Because measurements for the control group were below the LLOQ, a 2-sample t-test was performed between dosing groups 200 and 500 mg/kg. At peak expression, dosing group 500 mg/kg was significantly higher than dosing group 200 mg/kg ($p < 0.05$). At the 24 hour time-point, HO-1 levels decreased to 3,380 pg/mL and 1,870 pg/mL in dosing groups 200 and 500 mg/kg, respectively. Concentration of the protein continued to decrease to 1,137 pg/mL and 1,870 pg/mL in dosing groups 200 and 500 mg/kg, respectively, at the 36 hour time-point. Levels in both dosing groups fell below the LLOQ at the 48 hour time point. As discussed previously (Section 2.1), the profile data was normalized and graphed using either urine volume-weighted (Figure 35), creatinine-adjusted (Figure 36), or BACC baseline adjustments (Figure 37) for comparisons to HO-1 published data.

62
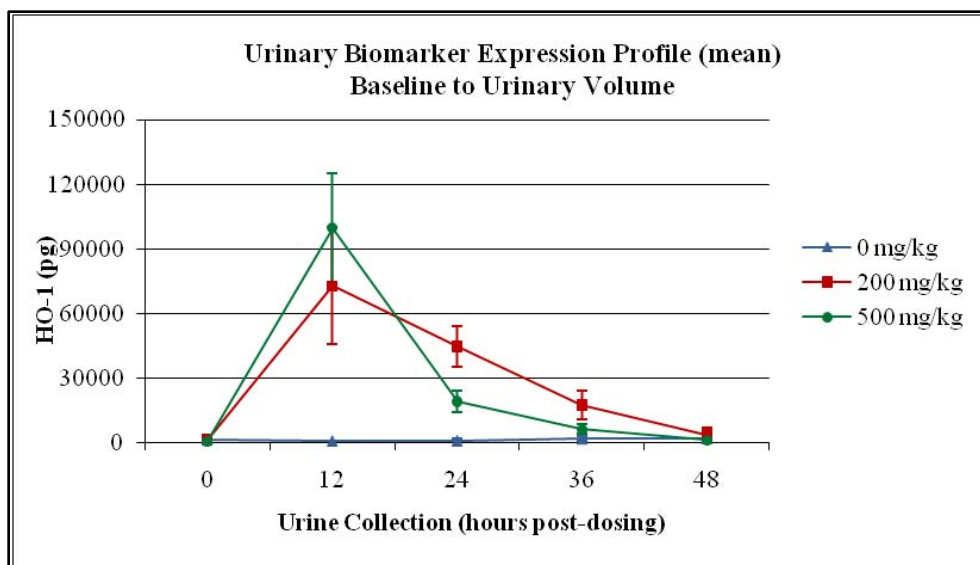
Figure 35: Measurement of urinary HO-1 (urine volume-weighted) by ELISA versus total urine volume/time point after D-serine administration (Study 2U)
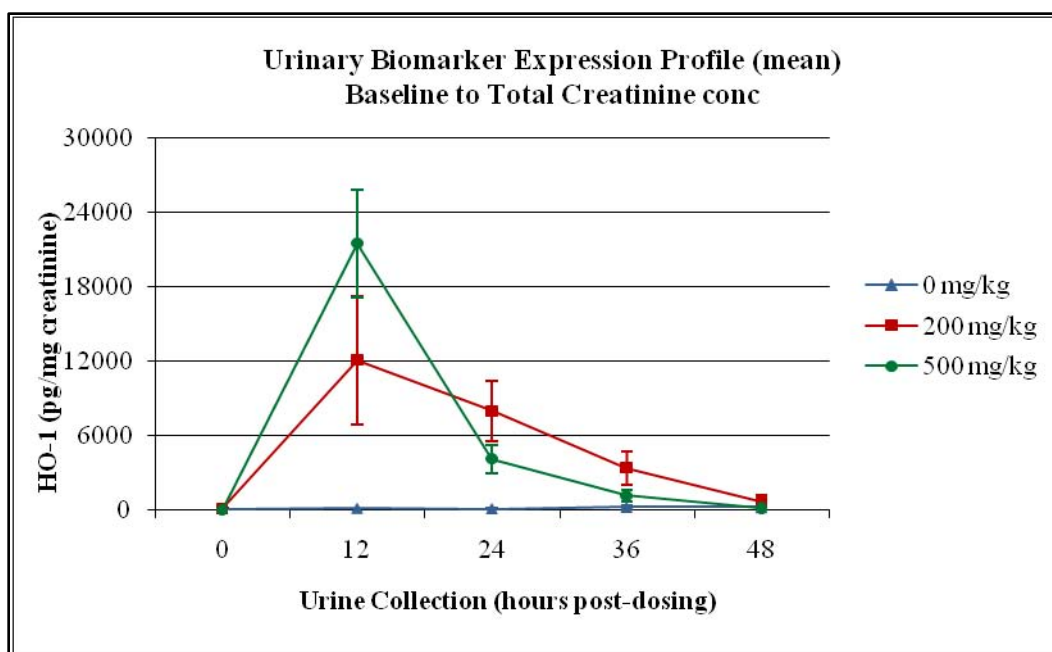
Figure 36: Measurement of urinary HO-1 (ng/mg creatinine) versus time point after D-serine administration (Study 2U)
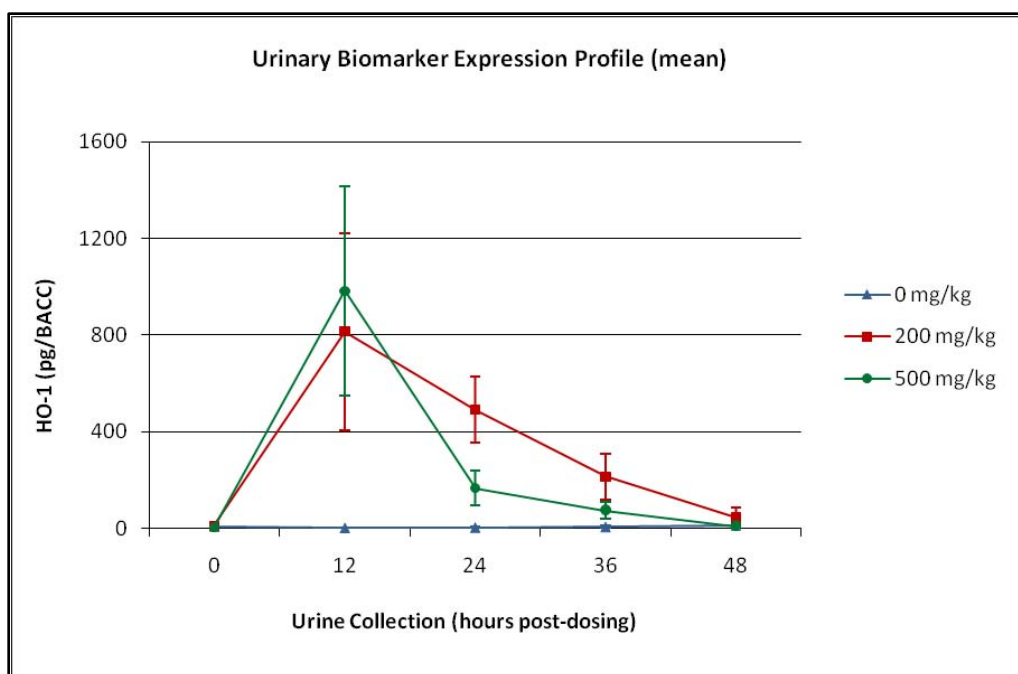
Figure 37: Measurement of urinary H (BACC adjusted) versus time point after D-serine administration (Study 2U)

HO-1 Profiling in rat serum samples from D-serine dosed animals.

<u>D-Serine Study 2S</u>

In order to determine whether changes in the HO-1 expression profile can be detected in blood prior to urine, the putative biomarker was quantitated in serum samples using ELISA. Male Fischer 344 rats were dosed with 200 or 500 mg/kg D-serine in saline, whereas control animals were dosed with saline alone. Ten animals were included in each dosing group. Five of the ten animals were sacrificed 12 hours post-dosing. The remaining five animals were sacrificed 24 hours post-dosing.

HO-1 levels of two out of the five 12-hour control animals fell below the LLOQ. The same was true for two out of the five 24-hour, 200 mg/kg-dosed animals, one out of the five 12-hour, 500 mg/kg-dosed animals and four out of the five 24-hour, 500 mg/kg-dosed animals. The serum data was analyzed with and without results that fell below the LLOQ. In addition, the data was analyzed with and without outliers (one of the five 12-hour control animals and one of the five 24-hour control animals). Neither two-way analysis of variance (ANOVA) nor a 2-sample t-test revealed a significant difference in HO-1 expression between the 12 and 24 hour time points of dosing groups 0, 200 and 500 mg/kg (Figure 38). Multiple statistical analyses also did not show significant differences between dosing groups at the two time points. Dilutions were adjusted accordingly to get samples to fall within the range of the assay.

64
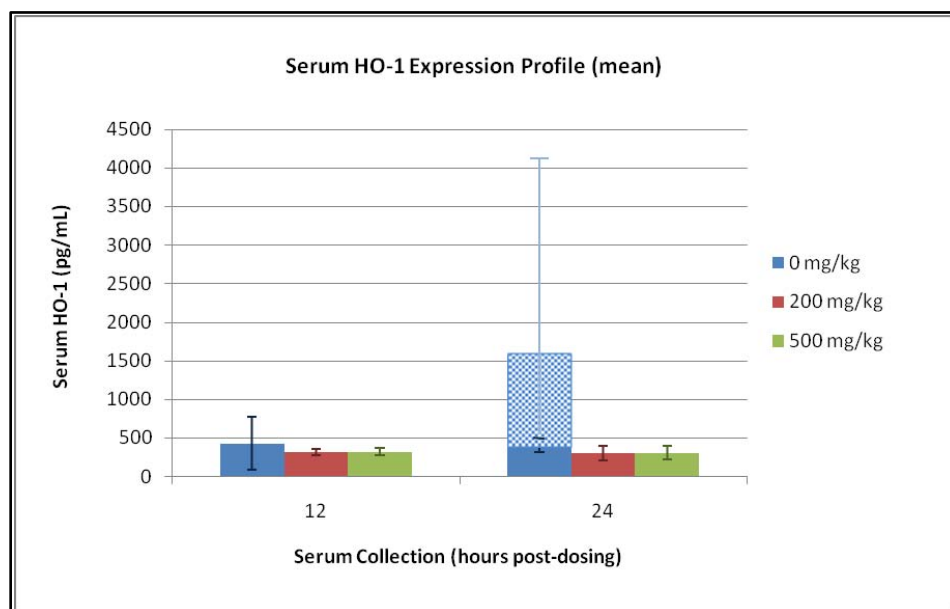
Figure 38: Measurement of serum HO-1 by ELISA at 12 h and 24 h after D-serine administration

2.6 α Glutathione-S-Transferase (α GST)

2.6.1 α GST Summary

Alpha glutathione-S-transferase (α GST) is a well characterized biomarker of renal injury in many kidney disease models, including acute kidney injury, and has been shown to be indicative of proximal tubular damage. Therefore, this protein was identified as a possible marker to be examined for its utility as an indicator of low level renal damage in a nephrotoxin model. To initiate subclinical kidney damage, several sub-lethal doses of the known nephrotoxin D-serine were used in a time/dose response study. Male Fischer 344 rats were dosed with 0, 5, 20, 50, 200, or 500 mg/kg D-serine in 0.9% saline, and urine was collected from animals pre-dose and 24, 48, 72 and 96 hours post-dose. All samples were frozen within 1 hour of end point collection. The urine samples were assayed for α GST levels using a validated rat α GST ELISA from Biotrin International Ltd. Quantitation of α GST samples post-dose indicated that its levels remained constant in animals dosed with 0, 5, 20, and 50 mg/kg D-serine throughout the time course. However, for animals dosed with 200 and 500 mg/kg D-serine, significant increases were observed at 24 hours post-dose, dropping to baseline (pre-dose) levels at 48 hours after nephrotoxin injection. The presence of α GST in the urine of the 200 and 500 mg/kg dose groups correlates with the histopathology data indicating the initiation of damage in the 200 mg/kg group. A second time-course experiment was conducted in order to confirm the peak response times and to clearly delineate the response time. In Study 2U, D-serine was again used to induce subclinical kidney damage at the proximal straight tubules using 200 or 500 mg/kg D-serine administered intraperitoneally. Urine samples were collected more frequently and for a longer time period than in Study 1, using 12 hour intervals during a 168 hour post-dose period with α GST again quantitated via ELISA. Using these samples, the α GST biomarker expression profile was further refined by identification of a significant increase occurring 12 hours post-dose before returning to baseline values, earlier than previously observed. Alpha GST was also examined for its usefulness as a biomarker in serum. To accomplish this, blood was collected at 12 and 24 hours post-dose animals dosed with 0, 200 and 500 mg/kg of D-serine, and α GST was quantitated by ELISA as before. Welch one-way ANOVA statistical analysis did not reveal significant differences in serum α GST levels between any of the dosing groups at 12 or 24 hours post-dose. The only statistical significance between dose groups/collection times was found in the 200 mg/kg dosing group evaluating α GST levels at 12 versus 24 hour post-dose. In summary, the reported data indicate that α GST is a responsive urinary biomarker to sub-clinical renal damage in the described nephrotoxin model, with an early and strongly detectible signal at 12 hours. Surprisingly, serum α GST was not indicative of kidney damage at any time point using any dose group, in contrast to published data in other injury models.

2.6.2 α GST Introduction

GST enzymes comprise a protein 'super family' that are primarily found in the cell cytosol. These proteins have been shown to catalyze conjugation of electrophilic substrates to glutathione, as well as exhibiting peroxidase and isomerase functions, inhibition of Jun N-terminal kinase, and non-catalytic binding to a wide range of cellular ligands. The ~24 Kdal mammalian GST proteins exist in the cytosol in dimer form, and are classified into alpha, mu, pi,

and theta classes based on several structural and enzymatic properties (review in Sheehan et al., 2001).

As a biomarker of kidney injury, α GST is one of the six best characterized renal injury biomarkers (NGAL, Kim-1, π GST, Clusterin, and β-2 microglobulin being the other five) and are currently used in preclinical animal studies during drug development for toxicity analysis. Urinary α GST levels have been shown to be responsive to kidney perturbation due to toxin exposures in a number of studies, including fluoride (Usuda et al., 1998), haematite and benzo pyrene (Boutin et al., 1998), and trichloroethylene (Brüning et al., 1999). Alpha GST, as well as Kim-1 and NAG, was shown to increase in the urine of Sprague-Dawley rats injected with multiple sub-lethal doses of cadmium (Prozialek et al., 2009). In this study, α GST did not appear in the urine until 8 weeks after treatment, although proteinuria was not present in the kidney until 10 weeks. In all toxin exposures, the mode of exposure was based on a chronic model or examining levels in humans for chronic toxin exposures, therefore the α GST urinary response from these studies may not translate to an acute exposure model.

In addition to its use as a toxicological exposure marker, α GST protein expression has been examined in the urine and serum in a number of renal diseases such as renal cell carcinoma (Grignon et al., 1994), renal damage in diabetes (Holmquist et al., 2008), and hypertension (Maarten et al., 1998) as well as hepatic injury (Beckett et al., 1987). Alpha GST also has been shown to be indicative of proximal tubular damage caused by proteinuria, a major cause of renal failure (Branten et al., 2000). Interestingly, in this study the highest urinary levels of α GST were found in patients with renal function, whereas π GST was expressed at the highest levels in patients with renal failure. It was also seen that urinary π GST inversely correlated with creatinine clearance while α GST did not. Using immunostaining, it was seen that α GST release into the urine correlated with brush border damage concurrent with a decrease expression (from control) in the proximal tubule. In the rat kidney α GST protein has been identified in the proximal and distal convoluted tubules which, when damaged, excrete this protein into the urine (Rozell et al., 1993; Kilty et al., 1998).

2.6.3 α GST Methods, Assumptions, and Procedures

Urine and Serum Collection

More detailed descriptions of the animal studies are described in *Biomarkers of Exposure to Toxic Substances, Vol 1* (DelRaso et al., Sep 2009). However, for clarity of this section, we include brief descriptions of each study.

D-serine Study 1

Male Fischer 344 rats were dosed with 5, 20, 50, 200 or 500 mg/kg D-serine in saline, whereas control animals were dosed with saline alone. Five animals were included in each dosing group with the exception of the 200 mg/kg group in which only four animals were included. Urine was collected in metabolic cages from animals prior to dosing and at 24, 48, 72

and 96 hours post-dose. One percent sodium azide, a bacteriostatic preservative, was added to each sample, and urine was frozen at -20°C within 1 hour of end point collection.

D-serine Study 2U (urine)

Male Fischer 344 rats were dosed with 200 or 500 mg/kg D-serine in saline, whereas control animals were dosed with saline alone. Five animals were included in each dosing group with the exception of the control group in which only four animals were included. Urine was collected from animals prior to dosing and at 12-hour intervals up to 168 hours post-dose. Protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) equal to 1/100 of the urine volume was added, and urine was frozen immediately at -20°C before being moved into a -80°C freezer within 1-7 days.

D-serine Study 2S (serum)

Male Fischer 344 rats were dosed with 200 or 500 mg/kg D-serine in saline, whereas control animals were dosed with saline alone. Ten animals were included in each dosing group. Five of the ten animals were sacrificed 12 hours post-dose. The remaining five animals were sacrificed 24 hours post-dose. Blood was collected from the inferior vena cava.

2.6.4 α GST Results and Discussions

α GST ELISA

Enzyme-linked immunosorbent assays (ELISA) were performed as described in the Biotrin rat α GST EIA kit protocol. First, urine samples were diluted 1:5 with sample diluent and serum samples were diluted 1:50 with sample diluent. One hundred microliters of diluted sample were added to the wells of 96-well microtiter plates coated with IgG directed against rat alpha GST and incubated for 1 hour at 20-25° C with uniform shaking. Plates were washed six times with wash solution (tris-buffered saline containing Tween 20). One hundred microliters of enzyme conjugate (anti-rat alpha GST IgG conjugated to horseradish peroxidase) were added and incubated for 1 hour at 20-25° C with uniform shaking. Wells were washed six times, 100 uL of substrate solution (stabilized tetramethylbenzidine solution) was added to the wells, and plates were incubated for 15 minutes at 20-25° C. Reactions were stopped by the addition of 100 uL of 1 N H_2SO_4. The absorbance (optical density [OD]) was measured at 450-630 nm with a microplate reader (Molecular Devices SpectraMax M2^{e}).

D-Serine Study 1 Urine Analysis

The known nephrotoxin D-serine was used to induce sub-clinical kidney damage at the proximal straight tubules in order to evaluate whether α GST could serve as an early predictor of nephrotoxicity. Doses of 20, 50, 200 or 500 mg/kg D-serine were administered intraperitoneally in 0.9% saline. Control animals were dosed with saline alone. Urine samples were collected at 24 hour intervals in a 96 hour period. α GST was quantitated by sandwich ELISA and the biomarker expression profile was determined.

Significant biomarker profile changes in α GST were seen in urine only in the 200 and 500 mg/kg D-serine dose groups. No significant profile changes were seen at the 5, 20 or 50 mg/kg D-serine dose groups (data not shown). Kidney histopathology data and clinical chemistry data (TBIL, CREA, BUN, and Total Protein) confirmed the initiation of kidney damage around the 200 mg/kg dose (data shown in DelRaso et al., Sep 2009). The urine profile of the biomarker peaked at 24 hours, reaching approximately 17,952 ug/L and 22,645 ug/L in dosing groups 200 and 500 mg/kg, respectively (Figure 39). At peak expression, dosing groups 200 and 500 mg/kg were significantly larger than the control group. However, the 200 and 500 mg/kg dosing groups were not significantly different from each other ($p<0.05$). At the 48 hour time-point, α GST levels decreased to as low as 281 ug/L and 359 ug/L in dosing groups 200 and 500 mg/kg, respectively. α GST levels continued to decrease at the 72 hour time point with levels of 29 ug/L and 82 ug/L in dosing groups 200 and 500 mg/kg, respectively. At the 96 hour time-point, α GST levels decreased to 47 ug/L in dosing group 500 mg/kg. However, in dose group 200 mg/kg, the α GST concentration remained at 30 ug/L, indicating a plateau in the excretion of the protein.
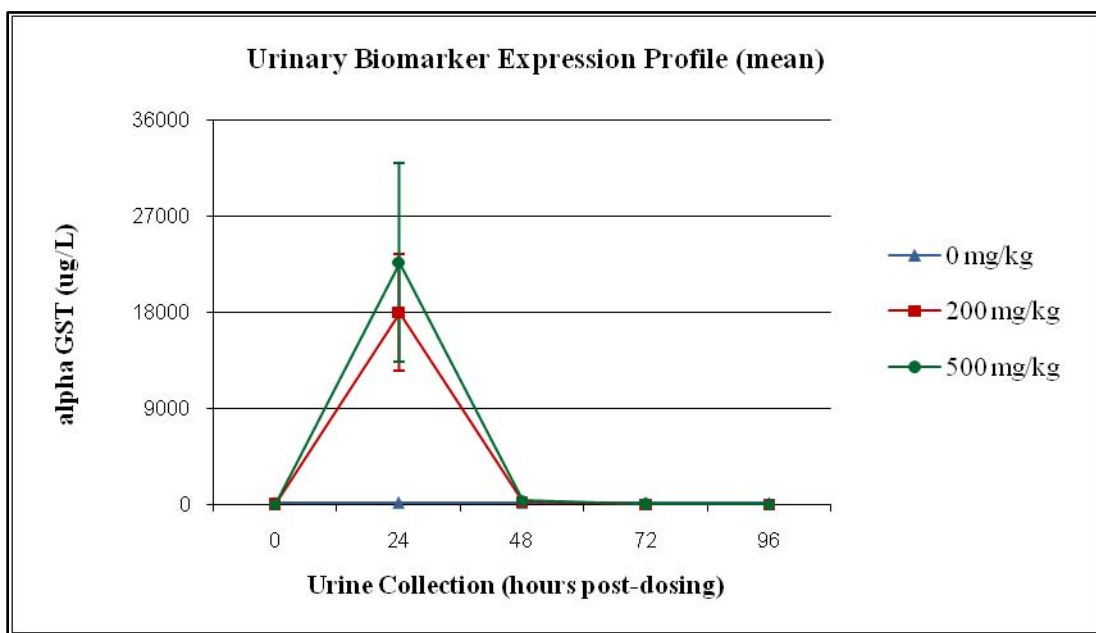
Figure 39: Measurement of urinary α GST by ELISA versus time after D-serine administration (Study 1)

D-Serine Study 2U Urine Analysis

A second time-course experiment was conducted in order to confirm that α GST peaks at 24 hours, not 12 or 36 hours. Again, D-serine was used to induce sub-clinical kidney damage at the proximal straight tubules. Doses of 200 or 500 mg/kg D-serine were administered intraperitoneally in 0.9% saline. Control animals were dosed with saline alone. Urine samples were collected at 12 hour intervals in a 168 hour period. α GST was quantitated by sandwich ELISA and the biomarker expression profile was determined.

As previously observed in the original time-course experiment, significant biomarker profile changes in α GST were seen in urine in the 200 and 500 mg/kg D-serine dose groups. Kidney histopathology data and clinical chemistry data (TBIL, CREA, BUN, and Total Protein) confirmed sub-clinical kidney damage at the 200 and 500 mg/kg doses (data not shown). Clarifying data from the previous study, the second time course experiment demonstrated the urine profile of the biomarker in fact peaks at 12 hours, reaching 28,566 ug/L and 39,351 ug/L in dosing groups 200 and 500 mg/kg, respectively (Figure 40).
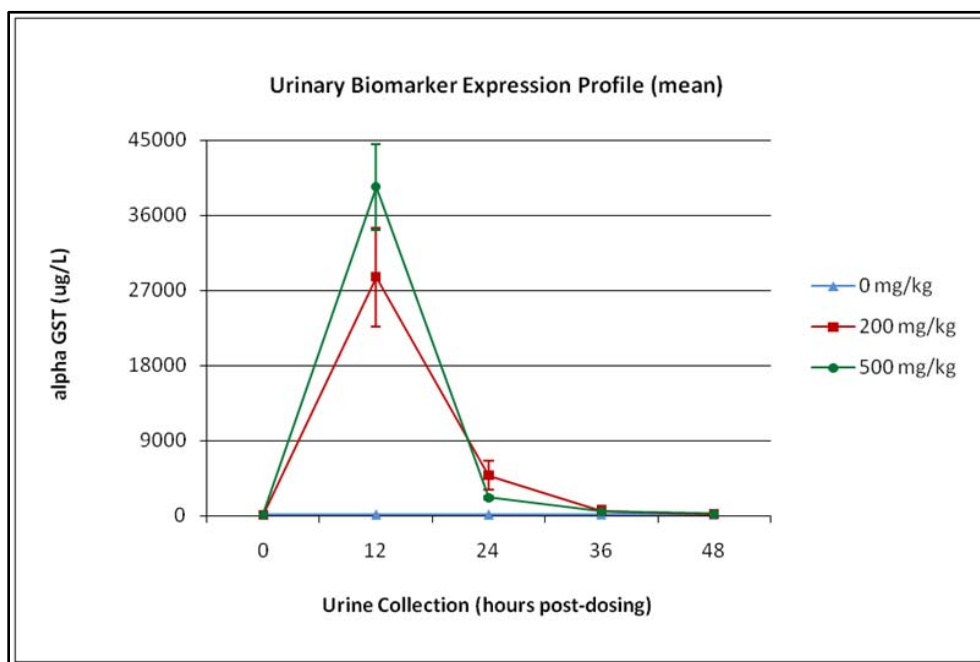
Figure 40: Measurement of urinary α GST versus time after D-serine administration (Study 2U)

A one-way ANOVA with weighted least squares to remedy a violation of assumption of equal variances, followed by multiple comparisons, was performed between dosing groups 0 mg/kg, 200 mg/kg and 500 mg/kg. At peak expression, a statistically significant difference ($p < 0.05$) was observed between all dosing groups with dosing group 500 mg/kg being the highest, followed by dosing group 200 mg/kg and dosing group 0 mg/kg. At the 24 hour time-point, α GST levels decreased to 4,794 ug/L and 2,155 ug/L in dosing groups 200 and 500 mg/kg, respectively. Concentration of the protein continued to decrease to 613 ug/L and 448 ug/L in dosing groups 200 and 500 mg/kg, respectively, at the 36 hour time-point. At the 48 hour time-point, α GST levels returned to control levels with concentrations of 174 ug/L and 187 ug/L in dosing groups 200 mg/kg and 500 mg/kg, respectively. As discussed previously (Section 2.1), the profile data was normalized and graphed using either urine volume-weighted (Figure 41), creatinine-adjusted (Figure 42), or BACC baseline adjustments (Figure 43) for comparisons to published α GST data.

70
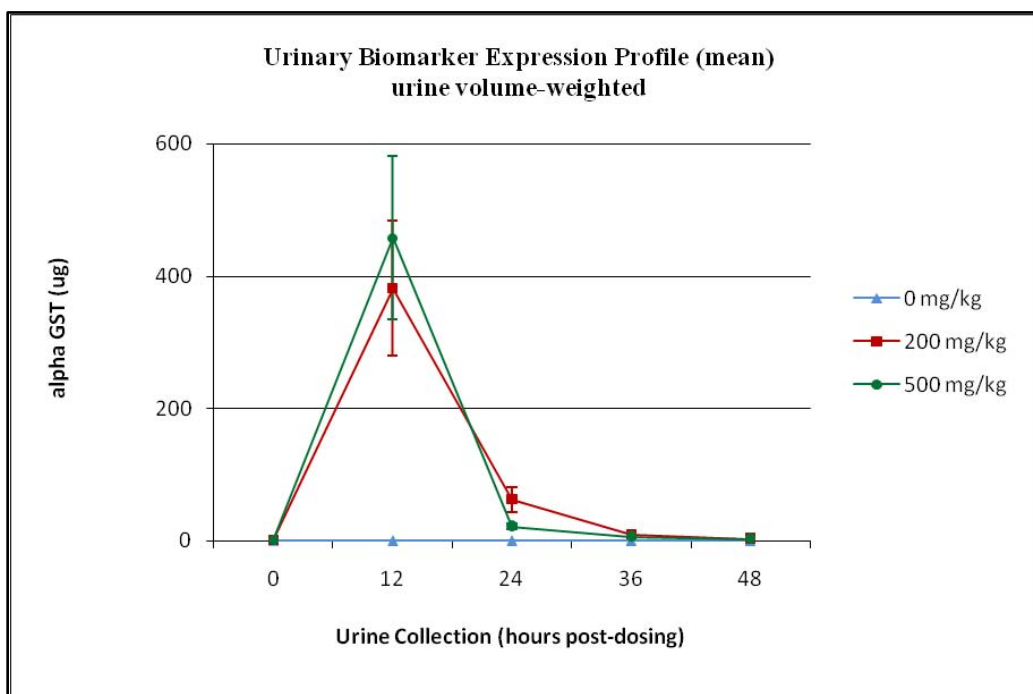
Figure 41: Measurement of urinary α GST (urine volume-weighted) versus time after D-serine administration (Study 2U)
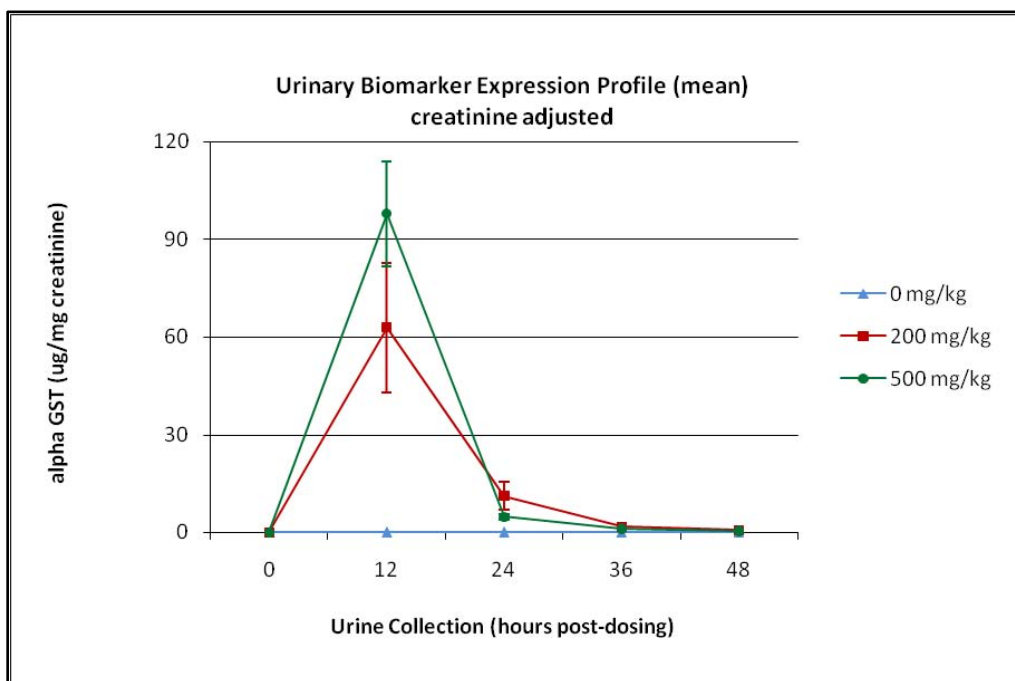
Figure 42: Measurement of urinary α GST (ng/mg creatinine) versus time point after D-serine administration (Study 2U)

71
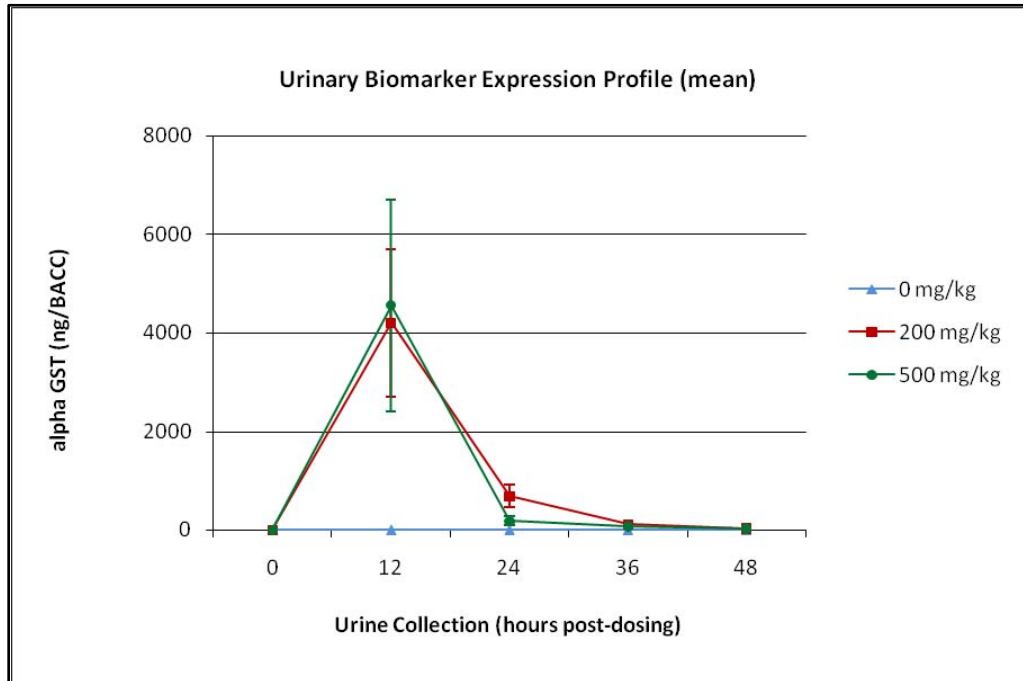
Figure 43: Measurement of urinary α GST (BACC adjusted) versus time point after D-serine administration (Study 2U)

D-Serine Study 2 Serum Analysis

In order to determine whether changes in the α GST expression profile can be detected in blood prior to urine, the putative biomarker was quantitated in serum samples using ELISA. Male Fischer 344 rats were dosed with 200 or 500 mg/kg D-serine in saline, whereas control animals were dosed with saline alone. Ten animals were included in each dosing group. Five of the ten animals were sacrificed 12 hours post-dosing. The remaining five animals were sacrificed 24 hours post-dosing.

A two-sample pooled t-test demonstrated that α GST expression did not differ significantly between the 12 and 24 hour time points of dosing groups 0 and 500 mg/kg. A significant difference in α GST expression between the two time points was observed in the 200 mg/kg dosing group, with expression at 12 hours being significantly higher. In the control group, the biomarker reached 1064 ug/L and 605 ug/L at the 12 and 24 hour time points, respectively (Figure 44). In the 200 mg/kg dosing group, α GST reached 1339 ug/L and 246 ug/L at the 12 and 24 hour time points, respectively. In the 500 mg/kg dosing group, α GST reached 1855 ug/L and 1260 ug/L at the 12 and 24 hour time points, respectively. A one-way ANOVA between dosing groups on 12 hour data showed no significant differences in the expression of α GST between the control and either dosing group or between the 200 and 500 mg/kg dosing groups. A Welch one-way ANOVA, which allows for different variances,

between dosing groups on 24 hour data also showed no significant differences in the expression of α GST between the control and either dosing group or between the 200 and 500 mg/kg dosing groups.

Figure 44: Measurement of serum α GST by ELISA at 12 h and 24 h after D-serine administration (Study 2S)

2.7 π Glutathione-S-Transferase (Yb1 GST)

2.7.1 Yb1 GST Summary

Pi GST (π GST) is a well characterized biomarker of both liver and renal injury, and expansive data in the literature demonstrate the utility of monitoring π GST levels in both serum and urine to determine damage levels to both organs. In addition, π GST levels also correlate to damage incurred upon toxin exposures, as well as renal damage from disease states. Studies on expression indicate that π GST in human, and the homolog Yb1 GST in the rat, are released in response to distal convoluted tubule, loop of Henle, or collecting duct damage in the kidney. As the published literature supported its use as a kidney injury marker, Yb1 GST was examined in a nephrotoxin rat model of subclinical kidney damage. D-serine was administered in sub-lethal doses to initiate very low levels of kidney degradation and the level of cellular damage was verified using histopathological examination of post-dose kidney tissues. In Study 1, male Fischer 344 rats were dosed with 0, 5, 20, 50, 200, or 500 mg/kg D-serine administered intraperitoneally in 0.9% saline and urine was collected from animals pre-dose and 24, 48, 72 and 96 hours post-dose. All samples were frozen within 1 hour of end point collection. The urine samples were assayed for Yb1GST levels using a validated rat Yb1 GST ELISA from Biotrin International Ltd. Yb1GST levels remained constant for control animals and in animals dosed with 5, 20, and 50 mg/kg D-serine throughout the post-dose period. The absence of marker response correlates with the lack of kidney cellular injury as seen in those dose groups. However, for dose groups 200 and 500 mg/kg D-serine which demonstrate low level injury, significant increases were observed with a significant peak at 24 hours post-dose. Yb1 GST levels dropped to baseline (pre-dose) levels after 48 hours. A second time-course experiment was conducted in order to confirm the peak response times of the protein. In Study 2U, D-serine was used to induce subclinical kidney damage at the proximal straight tubules using doses of 0, 200 or 500 mg/kg D-serine in 0.9% saline. Urine samples were collected at 12 hour intervals in a 168 hour period and frozen immediately, and Yb1 GST quantitated in the urine via ELISA. In Study 2U, it was seen that contrary to the previously observed 24 hour peak, peak levels of Yb1 GST were observed at 12 hours post-dose. Yb1 GST was not examined in serum. In light of this data, π GST (Yb1 GST) has been shown to be a responsive urinary biomarker to subclinical kidney injury due to toxin insult. However, as the D-serine nephrotoxin specifically generates injury in the proximal tubules and as π GST has been shown to be released in response to distal tubule injury, the mechanisms and cellular location of π GST release into the urine upon D-serine exposures remain to be determined.

2.7.2 Yb1 GST Introduction

Glutathione-S-transferases comprise a 'super family' of isozymes that vary in tissue expression and substrate specificities. In general, these proteins range from 17-28 kDal in size (Mantle et al., 1987) and are classified as alpha, mu, pi, or theta according to their structural and enzymatic properties (Sheehan et al., 2001). The π GST isozyme (24 kDal) has been shown to be a phase II detoxification cytosolic enzyme (Matsumoto et al., 1999) which inactivates some agents by conjugation with glutathione and subsequent removal by the multidrug resistance glycoprotein. The π GST is a homodimer of the Y(f) subunit, also known as the Y(b) in the rat (Campbell et al., 1991; Papp et al., 1995). Using immunostaining techniques, α GST expression

is found exclusively in the proximal tubules, while π GST is entirely absent. However, π GST is found in the distal convoluted tubules, the loop of Henle, as well as the collecting duct regions (Sundberg et al., 1994).

GST enzymes as renal injury markers were first studied in patients undergoing kidney transplant. It was shown that π GST was seen in the urine at a relatively constant baseline level (~ 6 ng/ml) while α GST cannot be detected. In patients experiencing kidney rejection π GST increased significantly while α GST levels were not altered, indicating that the primary damage in the kidney must be occurring at the distal segment. Research examining the effects of cyclosporine A demonstrated that urinary α GST levels increased upon dosing, whereas π GST remained constant (Sundberg et al., 1994) suggesting that the target of toxicity by cyclosporine A is at the proximal tubules. While cyclosporine A has been shown to target renal tubular epithelial cell damage by inducing cellular senescence (Jennings et al., 2007), it has yet to be shown if the distal as well as the proximal segments are affected. Studies with paracetamol (acetaminophen), a known nephrotoxin inducing apoptosis of proximal tubule epithelial cells (Lorz et al., 2004), have shown an increase in π GST (Marchewka et al., 2006). An examination of a low dose acute exposure of the nephrotoxin amphotericin B desoxycholate in humans indicated that π GST increased 2 to 4 fold and returned to baseline in the male, but was not altered in the female (Pai et al., 2005). This study provides some of the first data that sex-related differences may play a considerable role in baseline and response biomarker levels.

2.7.3 Yb1 GST Methods, Assumptions, and Procedures

Urine and Serum Collection

More detailed descriptions of the animal studies are described in *Biomarkers of Exposure to Toxic Substances, Vol 1* (DelRaso et al., Sep 2009). However, for clarity of this section, we include brief descriptions of each study.

D-serine Study 1

Male Fischer 344 rats were dosed with 5, 20, 50, 200 or 500 mg/kg D-serine in saline, whereas control animals were dosed with saline alone. Five animals were included in each dosing group with the exception of the 200 mg/kg group in which only four animals were included. Urine was collected in metabolic cages from animals prior to dosing and at 24, 48, 72 and 96 hours post-dose. One percent sodium azide, a bacteriostatic preservative, was added to each sample, and urine was frozen at -20°C within 1 hour of end point collection.

D-serine Study 2U (urine)

Male Fischer 344 rats were dosed with 200 or 500 mg/kg D-serine in saline, whereas control animals were dosed with saline alone. Five animals were included in each dosing group with the exception of the control group in which only four animals were included. Urine was collected from animals prior to dosing and at 12-hour intervals up to 168 hours post-dose. Protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) equal to 1/100 of the urine volume was added, and urine was frozen immediately at -20°C before being moved into a -80°C freezer within 1-7 days.

D-serine Study 2S (serum)

Male Fischer 344 rats were dosed with 200 or 500 mg/kg D-serine in saline, whereas control animals were dosed with saline alone. Ten animals were included in each dosing group. Five of the ten animals were sacrificed 12 hours post-dose. The remaining five animals were sacrificed 24 hours post-dose. Blood was collected from the inferior vena cava.

Yb1 GST ELISA Protocols

ELISAs were performed as described in the Biotrin GSTYb1 EIA - rat GST-mu kit protocol. First, urine samples were diluted 1:10 with sample diluent. One hundred microliters of diluted sample were added to the wells of 96-well microtiter plates coated with IgG directed against GSTYb1 and incubated for 1 hour at 20-25°C with uniform shaking. Plates were washed four times with wash solution (tris-buffered saline containing Tween 20). One hundred microliters of enzyme conjugate (anti-rat GSTYb1 IgG conjugated to horseradish peroxidase) were added and incubated for 1 hour at 20-25°C with uniform shaking. Wells were washed four times, 100 uL of substrate solution (stabilized liquid tetramethylbenzidine solution) was added to the wells, and plates were incubated for 15 minutes at 20-25°C. Reactions were stopped by the addition of 100 uL of 1 N H$_2$SO$_4$. The absorbance (optical density [OD]) was measured at 450-630 nm with a microplate reader (Molecular Devices SpectraMax M2^{e}).

2.7.4 Yb1 GST Results and Discussions

Examination of Yb1 GST in Urine samples from D-Serine Study 1

The known nephrotoxin D-serine was used to induce sub-clinical kidney damage at the proximal straight tubules in order to evaluate whether Yb1 GST could serve as an early predictor of nephrotoxicity. Doses of 20, 50, 200 or 500 mg/kg D-serine were administered intraperitoneally in 0.9% saline. Control animals were dosed with saline alone. Urine samples were collected at 24 hour intervals in a 96 hour period. Yb1 GST was quantitated by sandwich ELISA and the biomarker expression profile was determined.

The GST Yb1 EIA kit manual specifies a lower limit of detection (LLOD) of 0.2 ug/L in the microwell, equivalent to 2 ug/L in samples diluted 1:10. A limit of quantitation was not reported in the kit manual. For the purposes of this analysis, protein concentrations below that of the lowest calibrator (1.56 ug/L in the microwell) were considered below the lower limit of quantitation (LLOQ). Concentrations of samples that fell below the LLOQ were, never-the-less, calculated in order to perform statistical analysis between dosing groups. Protein levels in the control group fell below the LLOQ but above the LLOD at 24 hours, 48 hours and 72 hours for one out of the five rats. In addition, protein levels of one of the five rats in dosing group 500 mg/kg fell below the LLOQ but above the LLOD at 0 hours.

Significant biomarker profile changes in Yb1 GST were seen in urine only in the 200 and 500 mg/kg D-serine dose groups. No significant profile changes were seen at the 5, 20 or 50 mg/kg D-serine dose groups (data not shown). Kidney histopathology data and clinical chemistry data (TBIL, CREA, BUN, and Total Protein) confirmed the initiation of kidney

76

damage around the 200 mg/kg dose (data not shown). The urine profile of the biomarker peaked at 24 hours, reaching 801 ug/L and 1,199 ug/mL in dosing groups 200 and 500 mg/kg, respectively (Figure 45). At peak expression, dosing groups 200 and 500 mg/kg were significantly larger than the control group. However, the 200 and 500 mg/kg dosing groups were not significantly different from each other ($p < 0.05$). At the 48 hour time-point, Yb1 GST levels decreased to as low as 59 ug/L and 65 ug/L in dosing groups 200 and 500 mg/kg, respectively. Levels remained similar at the 72 hour time point, with Yb1 GST concentrations at 53 ug/L and 46 ug/L in dosing groups 200 and 500 mg/kg, respectively, and the 96 hour time point, with Yb1 GST concentrations at 16 ug/L and 37 ug/L in dosing groups 200 and 500 mg/kg, respectively. This indicates a plateau in the excretion of the protein.
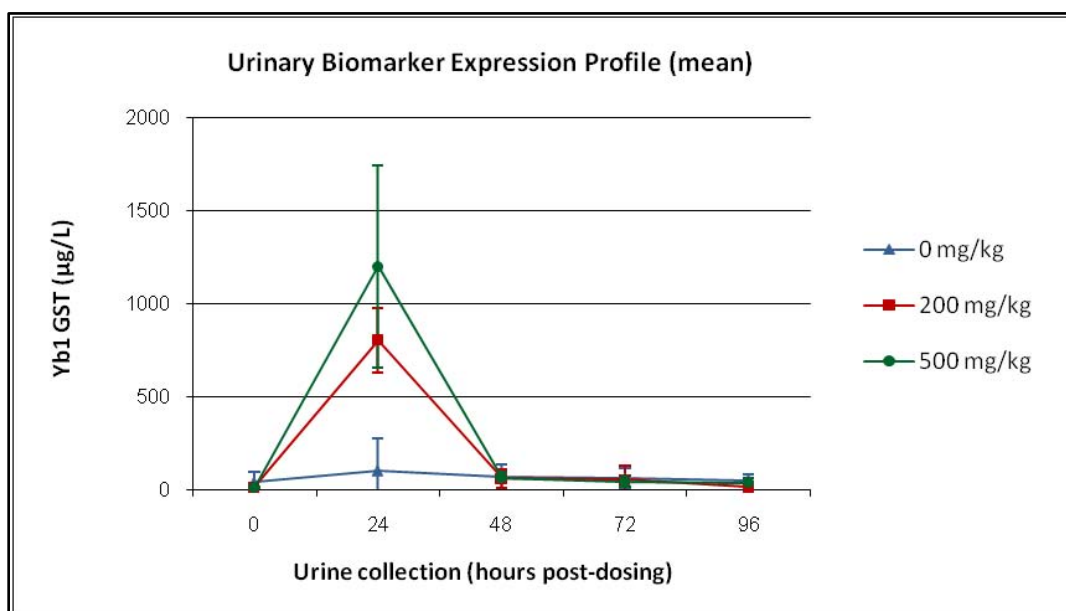
Figure 45: Measurement of urinary Yb1 GST by ELISA versus time after D-serine administration (Study 1)

Examination of Yb1 GST in Urine samples from D-Serine Study 2

A second time-course experiment was conducted in order to confirm that Yb1 GST peaks at 24 hours, not 12 or 36 hours. Again, D-serine was used to induce sub-clinical kidney damage at the proximal straight tubules. Doses of 200 or 500 mg/kg D-serine were administered intraperitoneally in 0.9% saline. Control animals were dosed with saline alone. Urine samples were collected at 12 hours intervals in a 168 hour period. Yb1 GST was quantitated by sandwich ELISA and the biomarker expression profile was determined.

As observed in Study 1, significant biomarker profile changes in Yb1 GST were seen in urine in the 200 and 500 mg/kg D-serine dose groups. Kidney histopathology data and clinical chemistry data (TBIL, CREA, BUN, and Total Protein) confirmed sub-clinical kidney damage at the 200 and 500 mg/kg doses (data shown in DelRaso et al., Sep 2009). Contrary to the previously observed 24 hour peak, the second time course experiment demonstrated the urine

77

profile of the biomarker in fact peaks at 12 hours, reaching 1,350 ug/L and 1,650 ug/L in dosing groups 200 and 500 mg/kg, respectively (Figure 46).
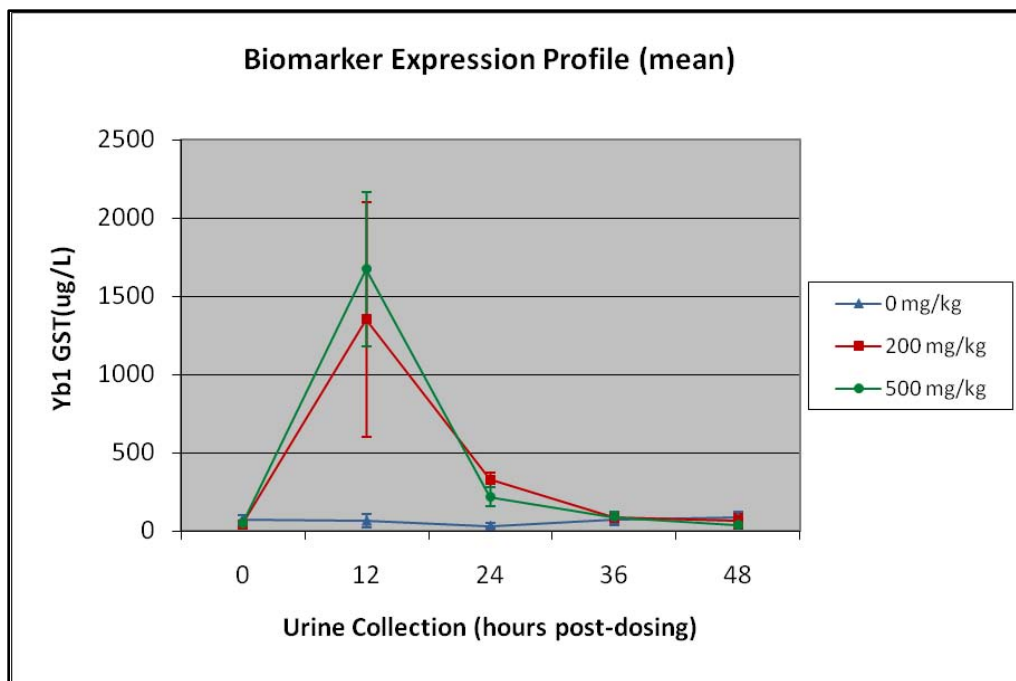
Figure 46: **Measurement of urinary Yb1 GST versus time after D-serine administration (Study 2U)**

As discussed previously (Section 2.1), the profile data was normalized and graphed using either urine volume-weighted (Figure 47), creatinine-adjusted (Figure 48), or BACC baseline adjustments (Figure 49) for comparisons to Yb1 GST published data. The differences seen between dose groups were examined to determine if these changes were statistically significant. In examining Yb1 GST differences in Study 2U, at 12 hour post-dose both the 200 and 500 mg/kg groups are statistically different than control using weighted least squares as a remedy for violation of assumption of equal variances. At 24 hours, all dose group differences are statistically significant. In examining creatinine-weighted Yb1GST values, only at 24 hour post-dose are statistically significant differences seen between control, 200, and 500 mg/kg D-serine animals. This statistically significance is also reflected in BACC adjusted Yb1 GST values. In examining urine-weighted values however, significant differences are seen in all three groups at 24 hours post-dose, but at 36 hours only the 200 mg/kg dose group differs significantly from control. The use of different weighted values thus can change significance levels (see Section 2.1) between dose groups.
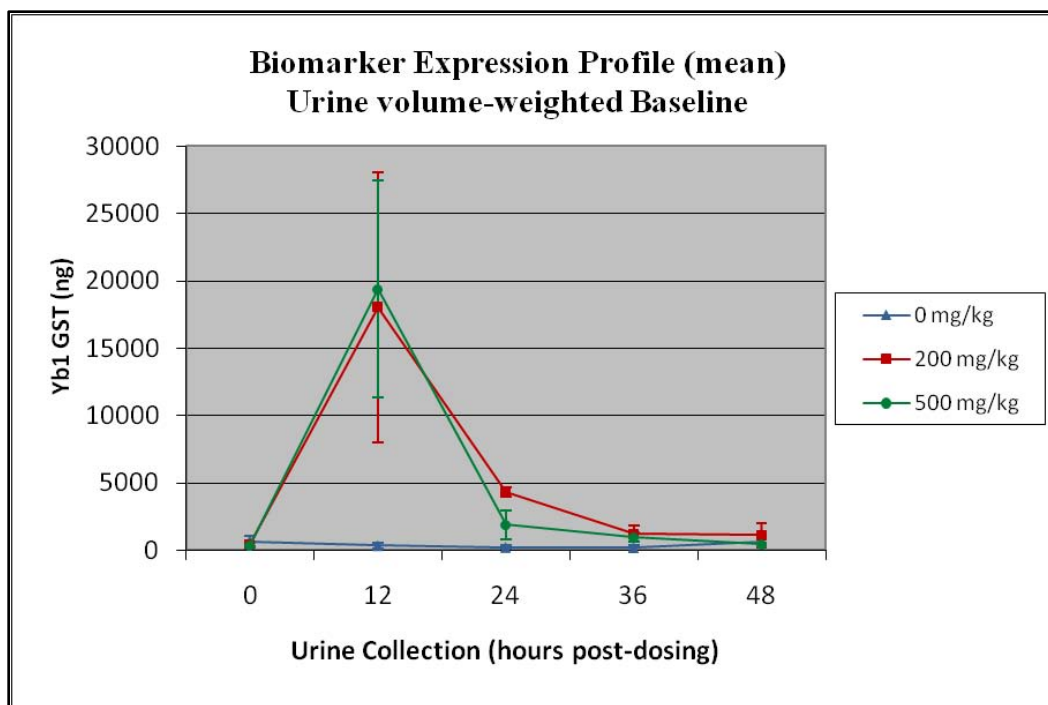
Figure 47: Measurement of urinary Yb1 GST (urine volume-weighted) versus time after D-serine administration (Study 2U)
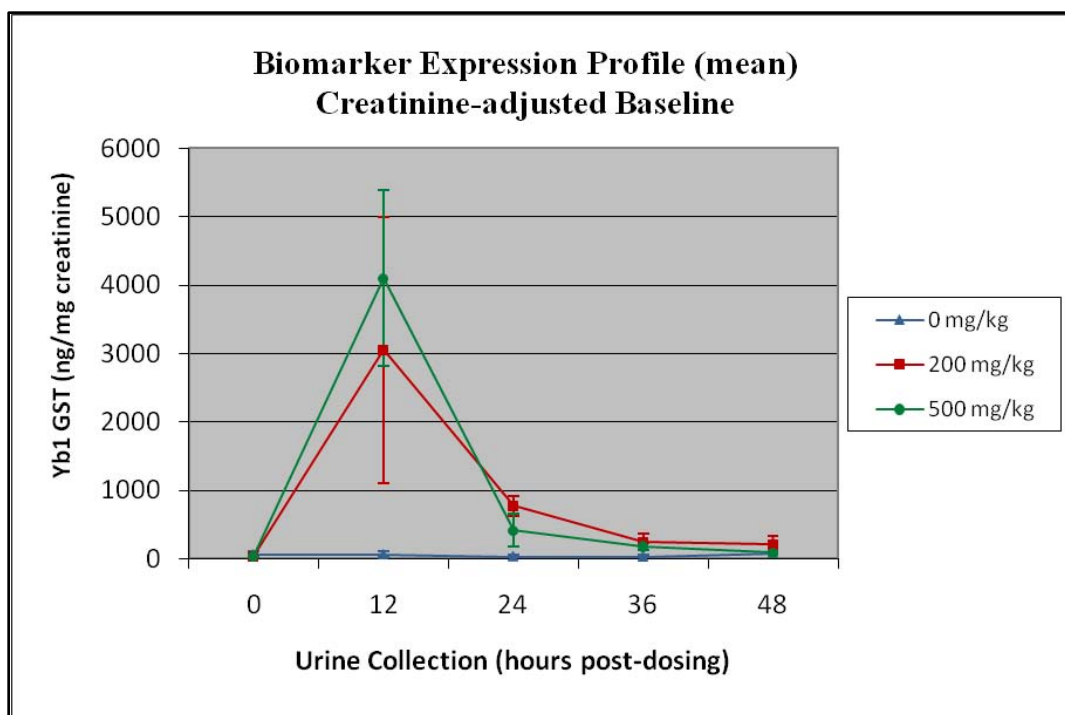
Figure 48: Measurement of urinary Yb1 GST (ng/mg creatinine) versus time point after D-serine administration (Study 2U)

79
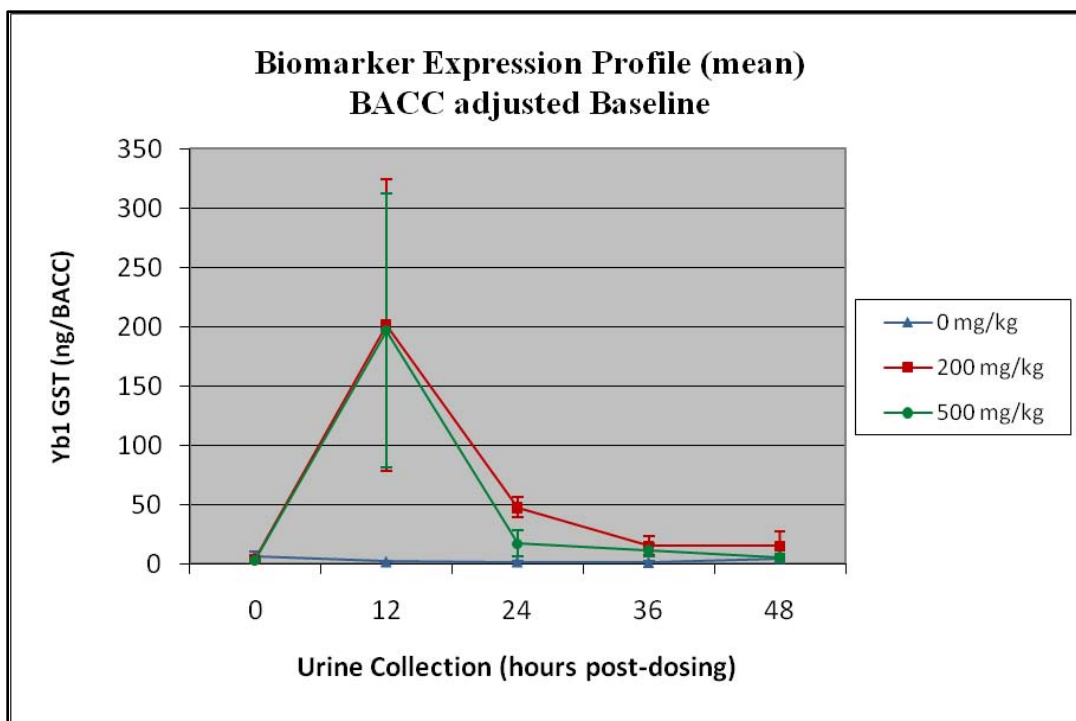
Figure 49: Measurement of urinary Yb1 GST (BACC adjusted) versus time point after D-serine administration (Study 2U)

2.8 β₂-Microglobulin (β2M)

2.8.1 β2M Summary

Current literature reveals that β_2-microglobulin (β2M) is a predictive biomarker of acute and chronic renal diseases, as well as injury due to specific chemical exposures. As this protein has demonstrated renal injury signaling capabilities, it was examined in a nephrotoxin model of subclinical kidney damage to determine if this marker was indicative of the initiation of cellular damage. To examine low levels of renal injury, the β2M expression profile was examined in urine samples in a time course/dose response study using several sub-lethal doses of the known nephrotoxin D-serine. Male Fischer 344 rats were dosed with 0, 5, 20, 50, 200, or 500 mg/kg D-serine in 0.9% saline administered intraperitoneally. Urine samples were collected from animals pre-dose and at 24, 48, 72 and 96 hours post-dose and frozen within 1 hour of end point collection. β2M urinary protein was quantitated using a Alpco Diagnostics ELISA specific for rat β_2-microglobulin. It was shown that β2M levels remained constant animals dosed with 0, 5, 20, and 50 mg/kg D-serine at all time points. Animals dosed with 200 and 500 mg/kg D-serine demonstrated a significant peak at 24 hours post-dose, with levels dropping 72 hours post-dose to baseline (pre-dose) levels. These data correlate with the histopathological examination of kidney tissue which demonstrated that observable levels of cellular damage were seen only in dose groups 200 and 500 mg/kg D-serine. A second time-course experiment was conducted in order to refine the peak response time. Again, D-serine was used to induce subclinical kidney damage at the proximal straight tubules using doses of 0, 200 or 500 mg/kg D-serine. In Study 2U, urine samples were collected more frequently at 12 hour intervals for a total of 168 hours. As observed in the original time course experiment, β_2-microglobulin levels peaked at 24 hours post-dose. In addition to assaying urine samples, serum samples were assayed by ELISA for β_2-microglobulin. Blood was collected at 12 and 24 hours post-dose in control animals and animals dosed with 200 mg/kg and 500 mg/kg of D-serine. In this study serum β_2-microglobulin levels were higher at 12 hours than 24 hours post-dose. At 12 hours post-dose, β_2-microglobulin levels were significantly higher in the control group than the 500 mg/kg dosing group, indicating that serum β2M seems to decrease, not increase, upon exposure. No statistically significant differences in β_2-microglobulin levels were observed between the other pairs of dosing groups. These data indicate that β_2-microglobulin is a responsive urinary biomarker to low level targeted proximal tubular injury. In the serum β_2-microglobulin levels seem to decrease upon exposure, but these expression changes may not be significant enough to be used reliably in the serum test matrix to monitor early and low levels of renal injury.

2.8.2 β2M Introduction

β2M is a non-glycosylated 11.8 kDal protein produced in all cells which express the MHC class I antigen (Harris et al., 1986). In addition to its bound allotypic determinants, β2M forms a noncovalently-bound subunit to the α chain of the MHC class I molecule. It is thought that β2M protein stabilizes the structure to allow MHC surface expression (Harris et al., 1986). β2M is proteolytically cleaved from the cell surface-bound MHC molecule by proteolysis, and enters the extracellular space and body fluids as a monomer. Once released, β2M is can be detected in the blood, as well as in cerebrospinal fluids, and saliva. The expression levels of β2M

can be stimulated by many types of liver and kidney injury, as well as cancer, rheumatoid arthritis, viral infections, and other chronic inflammatory diseases (Beorchia et al., 1981; Schuster et al., 1976).

β_2-microglobulin is a well characterized biomarker in both renal injury and toxicity models, and data indicate that, unlike creatinine, its levels are not altered by age, sex, or muscle mass (Filler et al., 2002; Bianchi et al., 2001). Due to its retention and aggregation during renal failure, β2M is a known causative agent of dialysis-related amyloidosis (Winchester et al., 2003). The levels of β2M in chronic renal failure cases before and after hemodialysis indicated that β2M had marked serum and urine increases independent of age, sex, or dialysis time variables (Acciardo et al., 1989). β_2-microglobulin was also seen to correlate with increases in serum creatinine, as well as inversely correlate with an improvement in the glomerular filtration rate. β_2-microglobulin has also been identified as an excellent urinary biomarker to acute renal allograft rejection (Oetting et al., 2006) as well as idiopathic membranous nephropathy (Branten et al., 2005).

The potential usefulness of β2M as a clinical marker for nephrotoxicity was examined as early as 1989 (Bernard et al., 1989). Environmental exposure studies monitoring the urine of workers exposed occupational levels of mercury, lead, or cadmium indicated that early detection of renal damage using the urinary β2M biomarker was successful in reversing early kidney damage. Workers were monitored and their exposure to toxic metals was limited on the job site when urinary β2M increased to levels at 1500 mg/g creatinine indicating mild tubular damage. The monitoring of urinary β2M allowed these low levels of renal injury to be reversed (Roels et al., 1999).

2.8.3 β2M Methods, Assumptions, and Procedures

Urine and Serum Collection

More detailed descriptions of the animal studies are described in *Biomarkers of Exposure to Toxic Substances, Vol 1* (DelRaso et al., Sep 2009). However, for clarity of this section, we include brief descriptions of each study.

D-serine Study 1

Male Fischer 344 rats were dosed with 5, 20, 50, 200 or 500 mg/kg D-serine in saline, whereas control animals were dosed with saline alone. Five animals were included in each dosing group with the exception of the 200 mg/kg group in which only four animals were included. Urine was collected in metabolic cages from animals prior to dosing and at 24, 48, 72 and 96 hours post-dose. One percent sodium azide, a bacteriostatic preservative, was added to each sample, and urine was frozen at -20°C within 1 hour of end point collection.

D-serine Study 2U(urine)

Male Fischer 344 rats were dosed with 200 or 500 mg/kg D-serine in saline, whereas control animals were dosed with saline alone. Five animals were included in each dosing group with the exception of the control group in which only four animals were included. Urine was

collected from animals prior to dosing and at 12-hour intervals up to 168 hours post-dose. Protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) equal to 1/100 of the urine volume was added, and urine was frozen immediately at -20°C before being moved into a -80°C freezer within 1-7 days.

D-serine Study 2S(serum)

Male Fischer 344 rats were dosed with 200 or 500 mg/kg D-serine in saline, whereas control animals were dosed with saline alone. Ten animals were included in each dosing group. Five of the ten animals were sacrificed 12 hours post-dose. The remaining five animals were sacrificed 24 hours post-dose. Blood was collected from the inferior vena cava.

β–2–Microglobulin ELISA Protocols

ELISAs were performed as described in the Alpco Diagnostics microglobulin β-2 (rat) EIA kit protocol. First, urine samples were diluted 1:200 with sample diluent and serum samples were diluted 1:500 with sample diluent. Three hundred microliters of wash buffer (phosphate-buffered saline containing 0.05% v/v Tween 20) were added to the wells of 96-well microtiter plates coated with anti-rat β_2-microglobulin antibody and incubated for 10 minutes at room temperature. The wash solution was aspirated and 100 uL of diluted sample were added to the wells and incubated for 2 hours at room temperature. Plates were washed three times with wash buffer. One hundred microliters of enzyme-labeled antibody (peroxidase-conjugated anti-rat β_2-microglobulin antibody) were added and incubated for 1 hour at room temperature. Wells were washed three times, 100 uL of chromogenic substrate solution (3,3',5,5'-tetramethylbenzidine in N,N-dimethylformamide) was added to the wells, and plates were incubated for 15 minutes at room temperature. Reactions were stopped by the addition of 50 uL of 1 M H_2SO_4. The absorbance (optical density [OD]) was measured at 450 nm with a microplate reader (Molecular Devices SpectraMax M2^e).

Statistical Analysis

All data were the average of duplicate determinations. The results were expressed as mean values ± standard deviations (SD). Statistical methodology included analysis of variance (H0: $\mu_1 = \mu_2 = \ldots = \mu_k$ versus H_a: $\mu_i = \mu_j$). When a significant difference was found at $\alpha = .05$, Tukey's multiple comparisons were performed to identify which dosing groups differed significantly. To ensure validity of the analysis of variance, the Kolmogorov-Smirnov test of normality and Bartlett's test for equal variances were performed on the residuals. Where normality was not justified, the Kruskal-Wallis test was performed in lieu of the analysis of variance, followed by Tukey's multiple comparisons as appropriate. When the assumption of equal variances failed, transformations were performed to stabilize the variance.

2.8.4 β2M Results and Discussions

Examination of β2M in Urine Samples from D-serine Study 1

D-Serine Study 1 Urine Analysis

The known nephrotoxin D-serine was used to induce sub-clinical kidney damage at the proximal straight tubules in order to evaluate whether β_2-microglobulin could serve as an early predictor of nephrotoxicity. Doses of 20, 50, 200 or 500 mg/kg D-serine were administered intraperitoneally in 0.9% saline. Control animals were dosed with saline alone. Urine samples were collected in 24 hour intervals for a total of 96 hours. Beta-2-microglobulin was quantitated by sandwich ELISA and the biomarker expression profile was determined.

Significant biomarker profile changes in β_2-microglobulin were seen in urine only in the 500 mg/kg D-serine dose group. No significant profile changes were seen at the 5, 20 and 50 mg/kg D-serine dose groups (data not shown) or the 200 mg/kg dose group. Kidney histopathology data and clinical chemistry data (TBIL, CREA, BUN, and Total Protein) indicated kidney damage initiated around the 200 mg/kg dose (data not shown). The urine profile of the biomarker peaked at 24 hours in dosing group 500 mg/kg, reaching approximately 4.9 ug/mL (Figure 50). In dosing group 200 mg/kg, the urine profile of the biomarker peaked at 48 hours, reaching approximately 2.1 ug/mL. At the 24 hour time-point, dosing group 500 mg/kg was significantly larger than the control group and dosing group 200 mg/kg. The 200 mg/kg dosing group was not significantly different from the control group ($p < 0.05$). At the 48 hour time-point, β_2-microglobulin levels decreased only slightly to 4.1 ug/mL in dosing group 500 mg/kg. Levels decreased more substantially at the 72 hour time point, reaching 1.4 ug/mL. Beta-2-microglobulin levels remained near 1.4 ug/mL at the 96 hour time-point, indicating a plateau in the excretion of the protein.
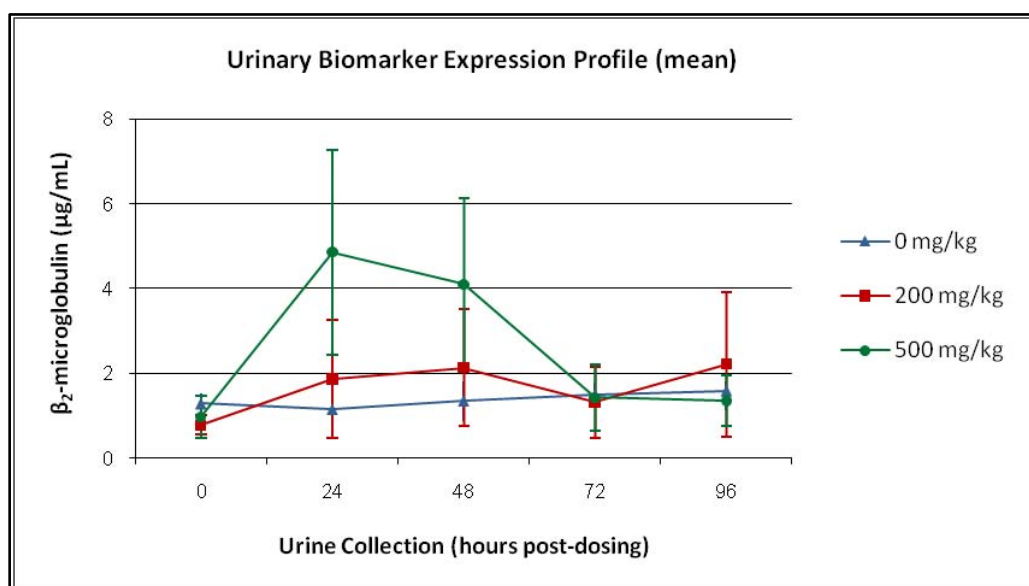
Figure 50: Measurement of urinary β_2-microglobulin by ELISA versus time after D-serine administration

D-Serine Study 2U Urine Analysis

A second time-course experiment was conducted in order to confirm that β_2-microglobulin peaks at 24 hours, not 12 or 36 hours. In addition, the experiment was designed to confirm a plateau in the excretion of the protein. Again, D-serine was used to induce sub-clinical kidney damage at the proximal straight tubules. Doses of 200 or 500 mg/kg D-serine were administered intraperitoneally in 0.9% saline. Control animals were dosed with saline alone. Urine samples were collected at 12 hour intervals in a 168 hour period. Beta-2-microglobulin was quantitated by sandwich ELISA and the biomarker expression profile was determined.

Unlike previously observed in the original time-course experiment, significant biomarker profile changes in β_2-microglobulin were seen in urine in the 200 and 500 mg/kg D-serine dose groups. Kidney histopathology data and clinical chemistry data (TBIL, CREA, BUN, and Total Protein) confirmed sub-clinical kidney damage at the 200 and 500 mg/kg doses (data not shown). In the 200 mg/kg dosing group, the highest concentration of β_2-microglobulin, 3298 ng/mL, was reached at 36 hours (Figure 51). However, this concentration was not significantly different from the concentration observed at the 24 hour time point. At 24 hours, dosing groups 200 and 500 mg/kg were significantly larger than the control group. However, the 200 and 500 mg/kg dosing groups were not significantly different from each other ($p < 0.05$). β_2-microglobulin levels measured at the 96 hour, 108 hour and 120 hour time points decreased to roughly 1400 ng/mL, 1200 ng/mL and 800 ng/mL in dosing groups 0 mg/kg , 200 mg/kg and 500 mg/kg, respectively. This suggests that the excretion of β_2-microglobulin plateaus at the later time points. As discussed previously (Section 2.1), the profile data was normalized and graphed using either

85

urine volume-weighted (Figure 52), creatinine-adjusted (Figure 53), or BACC baseline adjustments (Figure 54) for comparisons to β_2-microglobulin published data.
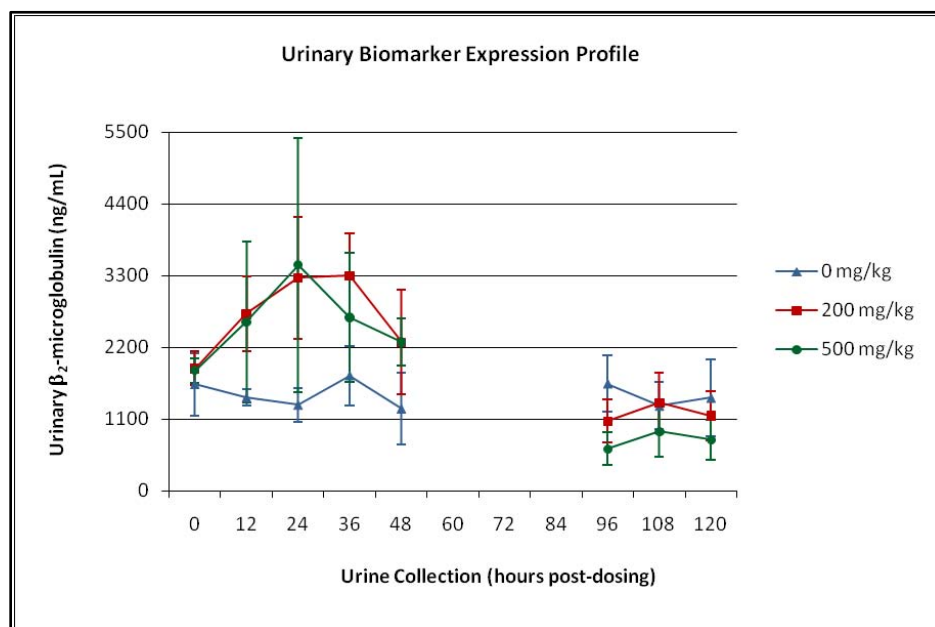
Figure 51: Measurement of urinary β_2-microglobulin by ELISA versus time after D-serine administration
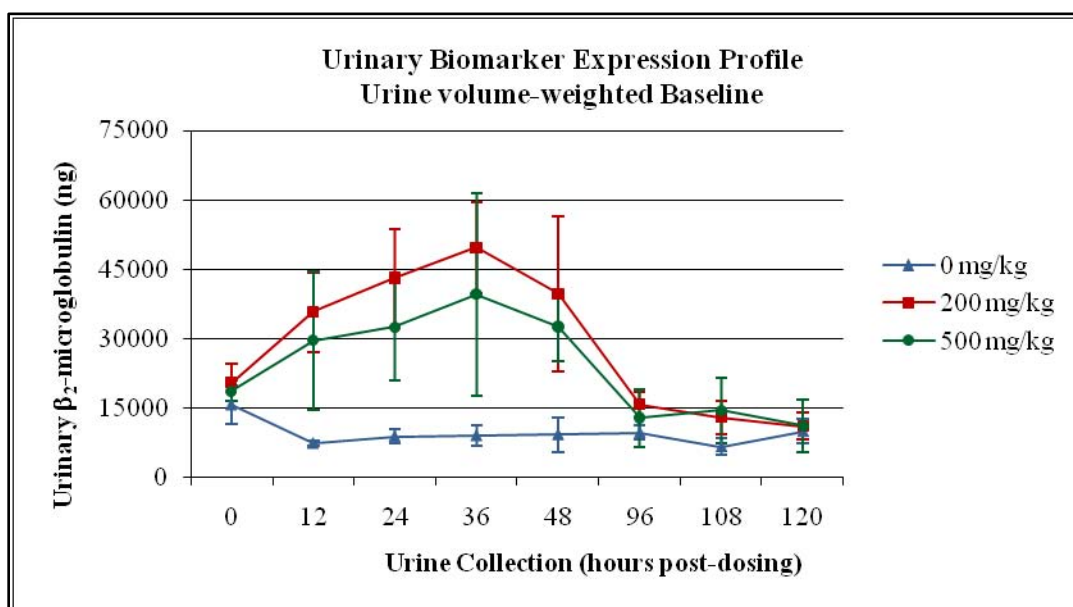
Figure 52: Measurement of urinary β_2-microglobulin(urine volume-weighted) versus time after D-serine administration (Study 2U)
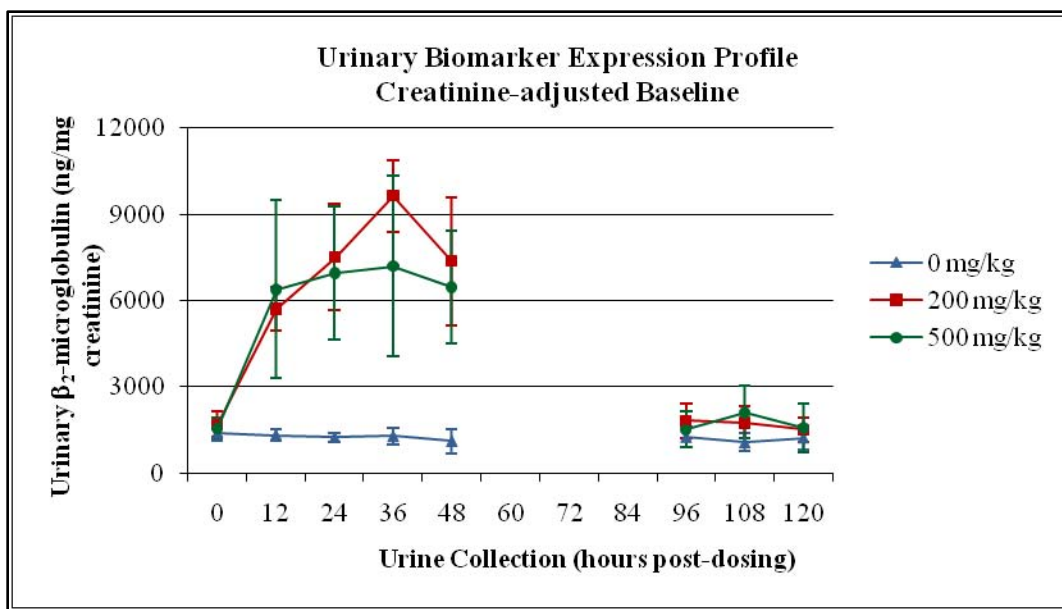
Figure 53: Measurement of urinary β₂-microglobulin (ng/mg creatinine) versus time point after D-serine administration (Study 2U)
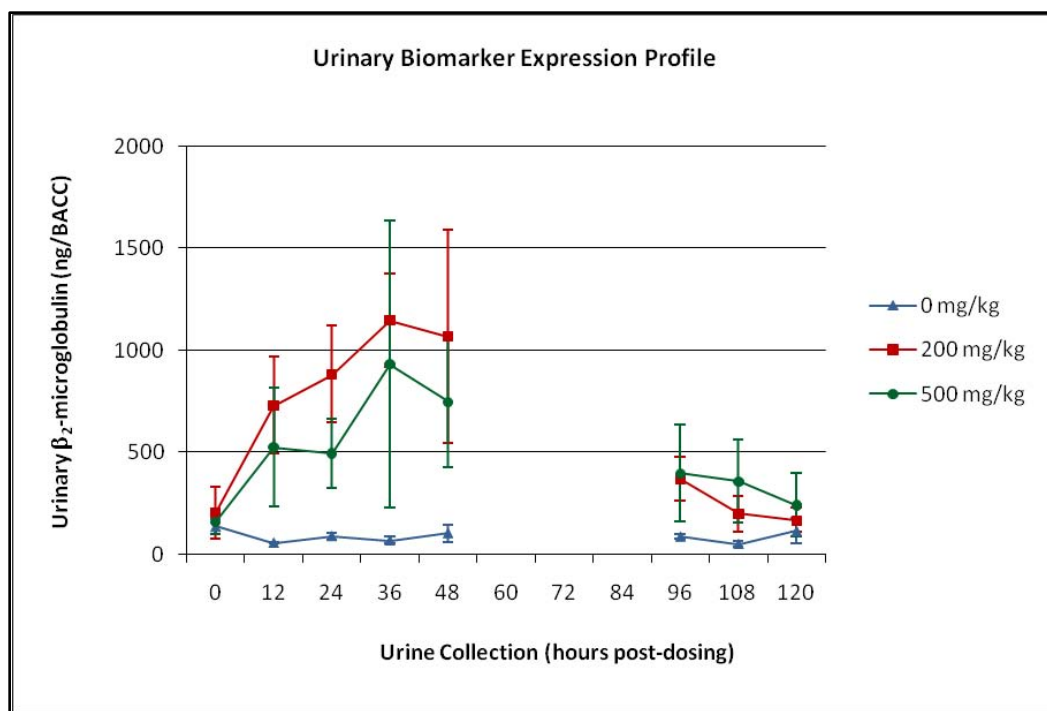
Figure 54: **Measurement of urinary β₂-microglobulin (BACC adjusted) versus time point after D-serine administration (Study 2U)**

No β₂-microglobulin analysis was conducted on samples taken between 48-96 hrs

87

In order to determine whether changes in the β_2-microglobulin expression profile can be detected in blood prior to urine, the putative biomarker was quantitated in serum samples using ELISA. Male Fischer 344 rats were dosed with 200 or 500 mg/kg D-serine in saline, whereas control animals were dosed with saline alone. Ten animals were included in each dosing group. Five of the ten animals were sacrificed 12 hours post-dosing. The remaining five animals were sacrificed 24 hours post-dose.

A two-way analysis of variance (ANOVA) showed that β_2-microglobulin expression peaked at the 12 hour time point (Figure 55). Beta-2-microglobulin levels reached 5,494 ng/mL, 4,823 ng/mL and 4,641 ng/mL in dosing groups 0 mg/kg, 200 mg/kg and 500 mg/kg, respectively. A one-way ANOVA revealed that expression of β_2-microglobulin in the control group was significantly higher than expression of the protein in the 500 mg/kg dosing group. A one-way ANOVA revealed no significant differences in β_2-microglobulin expression between the control group and the 200 mg/kg dosing group or between the 200 mg/kg and 500 mg/kg dosing groups.
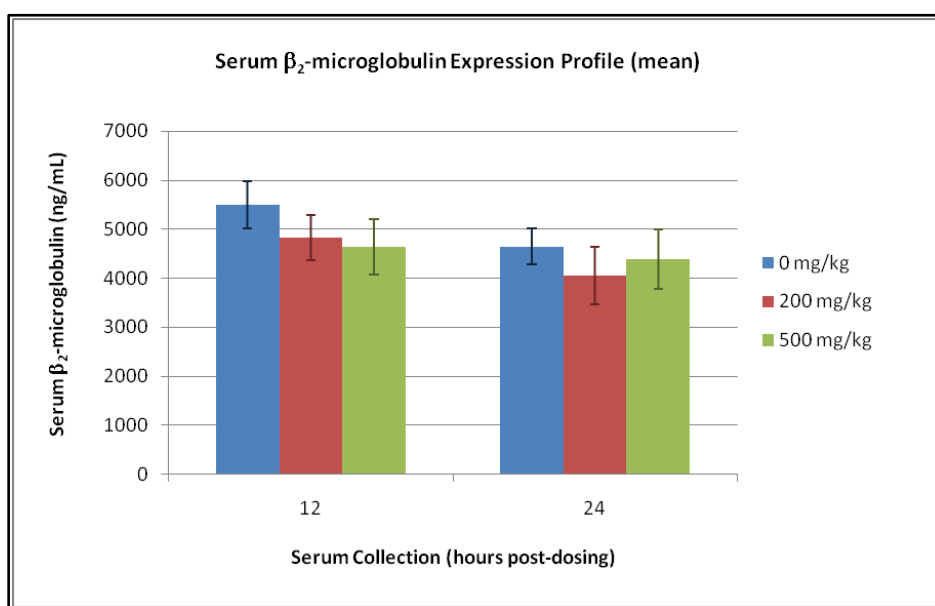
Figure 55: **Measurement of serum β_2-microglobulin by ELISA at 12 h and 24 h after D-serine administration**

2.9 Osteopontin (OPN)

2.9.1 OPN Summary

Osteopontin (OPN) was identified as a potential biomarker to renal injury through an extensive examination of published literature for proteins predictive of acute, chronic, chemically-induced, or disease-induced kidney damage. The protein expression profile of this protein was examined in urine in a time course response using several doses of the known nephrotoxin D-serine shown to induce subclinical renal damage. Male Fischer 344 rats were dosed with 0, 5, 20, 50, 200, or 500 mg/kg D-serine in saline, and the urine collected from animals prior to dosing and at 24, 48, 72 and 96 hours intervals post-dose. OPN was quantitated in the urine using the Assay Designs Rat Osteopontin Enzyme Immunometric kit. In this study it was seen that OPN levels remained constant for control animals and in animals dosed with 5, 20, and 50 mg/kg D-serine throughout the time course. However, for animals dosed with 200 and 500 mg/kg D-serine, significant increases were observed with peak response at 48 hours post-dose which dropped to baseline (pre-dose) levels at 72 hours. A secondary increase in OPN levels was also observed 96 hours post-dose. A second time-course experiment was conducted in order to confirm the OPN peak response times and to determine if this marker is indeed bimodal in nature. D-serine again used to induce sub-clinical kidney damage at the proximal straight tubules with doses of 0, 200 or 500 mg/kg administered intraperitoneally in 0.9% saline. In this second study, urine samples were collected at 12 hour intervals for a total of 168 hours and frozen immediately. Clarifying the previous data, OPN levels significantly increased at 36 hours post-dose. As previously observed, OPN levels dropped at 72 hours post-dose with marked increases observed at later time points, at 120 hours post-dose and 132 hours post-dose in dosing groups 200 mg/kg and 500 mg/kg, respectively. The presence of additional significant peaks at later time points may limit at its value as well as its inclusion on a renal biomarker panel. Nonetheless, urinary OPN was shown to respond in a quantitative, repeatable manner to D-serine exposures. Serum OPN levels were not tested due to the inability of the OPN ELISA to accurately quantitate in a serum test matrix. However, additional work with this marker using as-of-yet developed OPN ELISAs may indicate that OPN can function as a marker in the serum once a test kit is validated for quantitation in serum/plasma.

2.9.2 OPN Introduction

Osteopontin (OPN) is the principle phosphorylated glycoprotein of bone, but is expressed in many cell types, including renal tubular epithelium (Denhardt et al., 1993). OPN is produced in osteoblasts when stimulated by calcitriol, and is involved with the binding of osteoclasts to the mineral bone matrix via the vitronectin receptor. In the kidney, OPN is thought to influence the migration and attachment of immature tubular cells to the damaged basement membrane upon acute renal injury. OPN also plays a significant role in renal tissue remodeling due to the over expression of this protein during renal injury, an activity found as well as in other organs during injury requiring high cell turnover (Rodan et al., 1995).

Osteopontin has been shown to be over expressed in a number of cancer disease states, including cancers of the lung, breast, stomach, ovary, and skin. OPN knockout mice have demonstrated a reduced tolerance to renal ischemia, possibly by means of nitric oxide synthase

inhibition (Noiri et al., 1999). In the kidney, OPN mRNA and protein has been shown to increase upon acute nephrotoxicity in a rat model using mercuric chloride exposures to induce tubular injury and concomitant cell proliferation in the proximal straight tubules (Verstrepen et al., 2001). In the rat, increased OPN expression was also seen in angiotensin II hypertension (Giachelli et al., 1994), unilateral ureteral obstruction (Diamond et al., 1995), glomerulonephritis (Pichler et al., 1994), and tubulointerstitial nephritis (Eddy et al., 1995). OPN levels also increase upon cisplatin-induced injury (Iguchi et al., 2004) as well as during ethylene glycol induced calcium oxalate nephrolithiasis (Khan 2002). OPN has been found in high levels in the urine, where it is thought to inhibit calcium oxalate crystal formation (Min et al., 1998).

2.9.3 OPN Methods, Assumptions, and Procedures

Urine and Serum Collection

More detailed descriptions of the animal studies are described in *Biomarkers of Exposure to Toxic Substances, Vol 1* (DelRaso et al., Sep 2009). However, for clarity of this section, we include brief descriptions of each study.

D-serine Study 1

Male Fischer 344 rats were dosed with 5, 20, 50, 200 or 500 mg/kg D-serine in saline, whereas control animals were dosed with saline alone. Five animals were included in each dosing group with the exception of the 200 mg/kg group in which only four animals were included. Urine was collected in metabolic cages from animals prior to dosing and at 24, 48, 72 and 96 hours post-dose . One percent sodium azide, a bacteriostatic preservative, was added to each sample, and urine was frozen at -20°C within 1 hour of end point collection.

D-serine Study 2U(urine)

Male Fischer 344 rats were dosed with 200 or 500 mg/kg D-serine in saline, whereas control animals were dosed with saline alone. Five animals were included in each dosing group with the exception of the control group in which only four animals were included. Urine was collected from animals prior to dosing and at 12-hour intervals up to 168 hours post-dose. Protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) equal to 1/100 of the urine volume was added, and urine was frozen immediately at -20°C before being moved into a -80°C freezer within 1-7 days.

D-serine Study 2S(serum)

Male Fischer 344 rats were dosed with 200 or 500 mg/kg D-serine in saline, whereas control animals were dosed with saline alone. Ten animals were included in each dosing group. Five of the ten animals were sacrificed 12 hours post-dose. The remaining five animals were sacrificed 24 hours post-dose. Blood was collected from the inferior vena cava.

Osteopontin ELISA

ELISAs were performed as described in the Assay Designs rat osteopontin Enzyme Immunometric Assay kit protocol. First, urine samples were diluted 1:500 with assay buffer (phosphate-buffered saline containing proteins and detergents). One hundred microliters of diluted sample were added to the wells of 96-well microtiter plates coated with rabbit antibody specific to rat osteopontin (OPN) and incubated for 1 hour at 37° C. Plates were washed seven times with wash buffer (phosphate-buffered saline containing detergents). One hundred microliters of labeled antibody conjugate (rabbit antibody to rat OPN conjugated to horseradish peroxidase) were added and incubated for 30 minutes at 4° C. Wells were washed nine times, 100 uL of substrate solution (3,3',5,5'-tetramethylbenzidine and hydrogen peroxide) was added to the wells, and plates were incubated for 30 minutes at room temperature in the dark. Reactions were stopped by the addition of 100 uL of 1 N H_2SO_4. The absorbance (optical density [OD]) was measured at 450-570 nm with a microplate reader (Molecular Devices SpectraMax M2^{e}).

Statistical Analysis

All data were the average of duplicate determinations. The results were expressed as mean values ± standard deviations (SD). Statistical methodology included analysis of variance (H0: $\mu_1 = \mu_2 = \ldots. = \mu_k$ versus H_a: $\mu_i = \mu_j$). When a significant difference was found at $\alpha = .05$, Tukey's multiple comparisons were performed to identify which dosing groups differed significantly. To ensure validity of the analysis of variance, the Kolmogorov-Smirnov test of normality and Bartlett's test for equal variances were performed on the residuals. Where normality was not justified, the Kruskal-Wallis test was performed in lieu of the analysis of variance, followed by Tukey's multiple comparisons as appropriate. When the assumption of equal variances failed, transformations were performed to stabilize the variance.

2.9.4 OPN Results and Discussions

D-Serine Study 1 Urine Analysis

The nephrotoxin D-serine was used to induce sub-clinical kidney damage at the proximal straight tubules in order to evaluate whether osteopontin could serve as an early predictor of nephrotoxicity. Doses of 20, 50, 200 or 500 mg/kg D-serine were administered intraperitoneally in 0.9% saline. Control animals were dosed with saline alone. Urine samples were collected at 24 hour intervals in a 96 hour period. Osteopontin was quantitated by sandwich ELISA and the biomarker expression profile was determined.

The osteopontin EIA kit manual specified a lower limit of detection (LLOD) of 19.5 pg/mL. A limit of quantitation was not reported in the kit manual. For the purposes of this analysis, protein concentrations below that of the lowest calibrator (74 pg/mL) and above the LLOD were calculated and included in the statistical analysis. Samples with concentrations that fell below the LLOD were reported as BDL (below detection limit) and excluded from statistical analysis. Protein levels of the following rats fell below the LLOD: four out of five rats in the control group at 0 and 72 hours, three out of five rats in the control group at 24 and 48 hours, two

91

out of five rats in the control group at 96 hours, one out of four rats in the 200 mg/kg dosing group at 0, 24 and 72 hours, two out of five rats in the 500 mg/kg dosing group at 0 and 72 hours, all rats in the 500 mg/kg dosing group at 24 hours, one out of five rats in the 500 mg/kg dosing group at 48 and 96 hours.

Significant biomarker profile changes in osteopontin were seen in urine only in the 200 mg/kg D-serine dose group. No significant profile changes were seen at the 5, 20 and 50 mg/kg D-serine dose groups (data not shown) or the 500 mg/kg dose group. Kidney histopathology data and clinical chemistry data (TBIL, CREA, BUN, and Total Protein) indicated kidney damage initiated around the 200 mg/kg dose (data not shown). An increase in the concentration of urinary osteopontin was first observed at 48 hours, with levels reaching 413 ng/mL and 166 ng/mL in dosing groups 200 and 500 mg/kg, respectively (Figure 56). At that time point, dosing group 200 mg/kg was significantly larger than the control group and dosing group 500 mg/kg (p<0.05). The control group and 500 mg/kg dosing group were not significantly different from each other. At the 72 hour time-point, osteopontin levels decreased to 137 ng/mL in dosing groups 200 and 500 mg/kg. The urine profile of the biomarker peaked at 96 hours, with osteopontin concentrations reaching 433 ng/mL and 334 ng/mL in dosing groups 200 and 500 mg/kg, respectively. No plateau was observed in the excretion of osteopontin during the 96 hour time-course. Instead, osteopontin may exhibit a biphasic expression profile. A longer time-course experiment extending to 168 hours was conducted to clarify any relevancy and extent of the 96 hour peak.
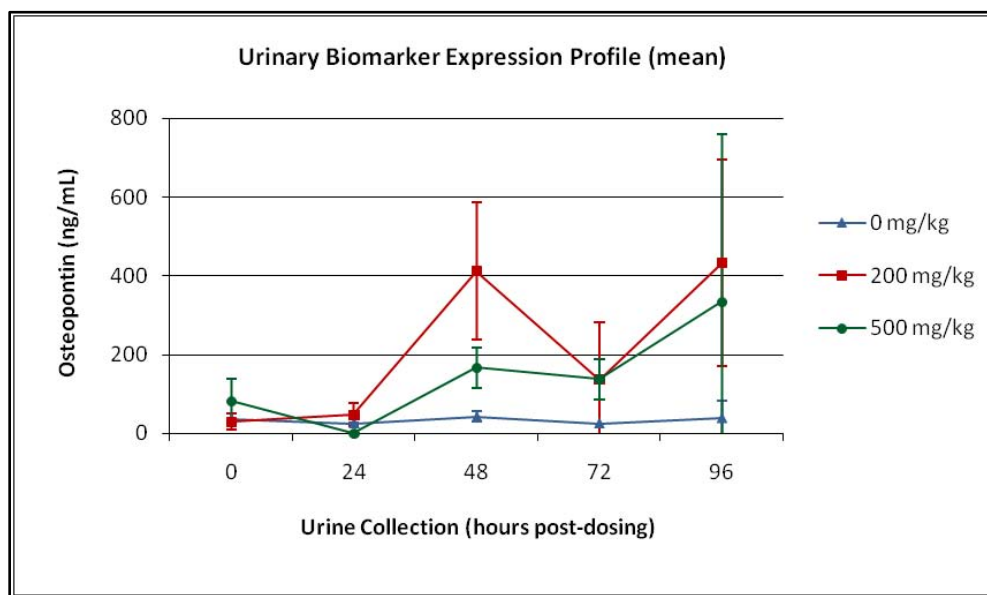
Figure 56: Measurement of urinary osteopontin by ELISA versus time after D-serine administration (Study 1)

A second time-course experiment was conducted in order to clarify the relevancy of the increase in osteopontin concentration at the 96 hour time point as well as to confirm that the potential biomarker marker peaks at 48 hours. Again, D-serine was used to induce sub-clinical

92

kidney damage at the proximal straight tubules. Doses of 200 or 500 mg/kg D-serine were administered intraperitoneally in 0.9% saline. Control animals were dosed with saline alone. Urine samples were collected at 12 hours intervals in a 168 hour period. Osteopontin was quantitated by sandwich ELISA and the biomarker expression profile was determined.

As in the original time-course experiment, protein concentrations below that of the lowest calibrator (74 pg/mL) and above the LLOD (19.5 pg/mL) were calculated and included in the statistical analysis. Samples with concentrations that fell below the LLOD were reported as BDL (below detection limit) and excluded from statistical analysis. Protein levels of the following rats fell below the LLOD: one out of four rats in the control group at 0, 12, 48, 84, 144 and 168 hours, two out of four rats in the control group at 96 hours, one out of five rats in the 200 mg/kg dosing group at 0 and 12 hours, one out of five rats in the 500 mg/kg dosing group at 72 hours.

Unlike previously observed in the original time-course experiment, significant biomarker profile changes in osteopontin were seen in urine in the 200 and 500 mg/kg D-serine dose groups. Kidney histopathology data and clinical chemistry data (TBIL, CREA, BUN, and Total Protein) confirmed sub-clinical kidney damage at the 200 and 500 mg/kg doses (data not shown). Contrary to the previously observed 48 hour peak, the second time course experiment demonstrated the urine profile of the biomarker in fact first peaks at 36 hours, reaching 995 ng/mL and 1,165 ng/mL in dosing groups 200 and 500 mg/kg, respectively (Figure 57).
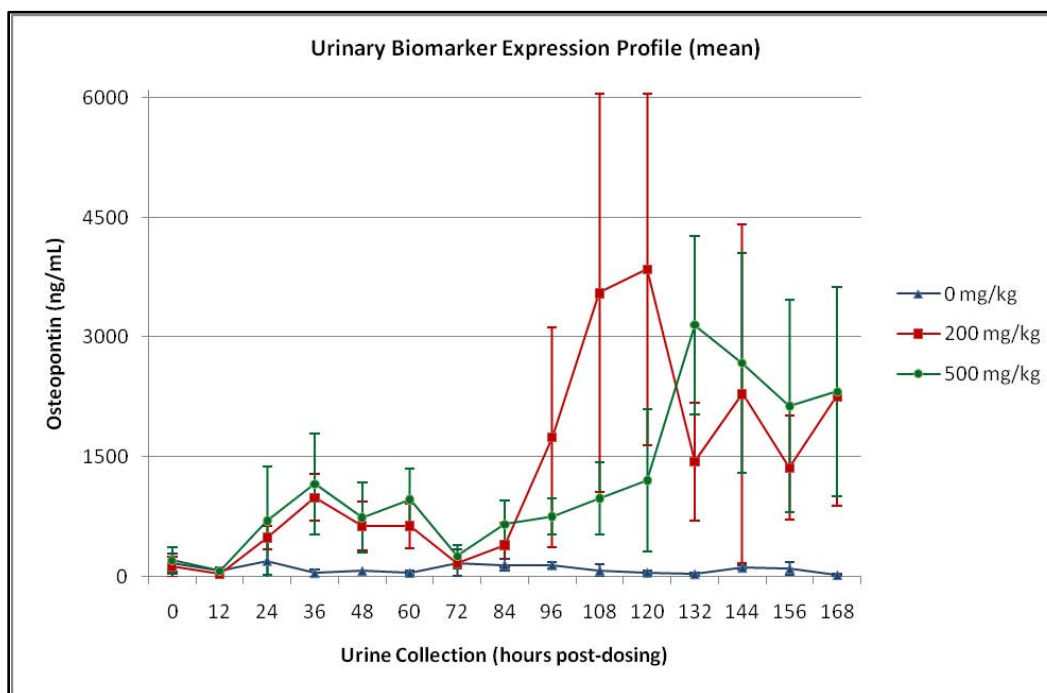
Figure 57: Measurement of urinary OPN versus time after D-serine administration (Study 2U)

At 36 hours, osteopontin concentrations in dosing groups 200 mg/kg and 500 mg/kg were significantly higher than concentrations in the control group (p<0.05). Dosing groups 200 mg/kg and 500 mg/kg were not significantly different from each other. A more pronounced increase in osteopontin concentration was observed at the later time points. More specifically, expression in the 200 mg/kg dosing group peaked at 120 hours and expression in the 500 mg/kg dosing group peaked at 132 hours. At those time-points, osteopontin levels reached 3,851 ng/mL and 3,152 ng/mL in dosing groups 200 and 500 mg/kg, respectively. Osteopontin concentrations in the control group were significantly lower than in dosing groups 200 mg/kg and 500 mg/kg, but dosing groups 200 mg/kg and 500 mg/kg did not differ significantly from each other. Concentrations of the protein remained elevated for the remainder of the time-course experiment and did not fall below 1,366 ng/mL and 2,140 ng/mL in dosing groups 200 and 500 mg/kg, respectively. As discussed previously (Section 2.1), the profile data was normalized and graphed using either urine volume-weighted (Figure 58), creatinine-adjusted (Figure 59), or BACC baseline adjustments (Figure 60) for comparisons to OPN published data. Interestingly, the 200 mg/kg dose group seems to exhibit an OPN peak 12 hours prior to that seen with the 500 mg/kg group, an observation that is consistent with all methods of data normalization.
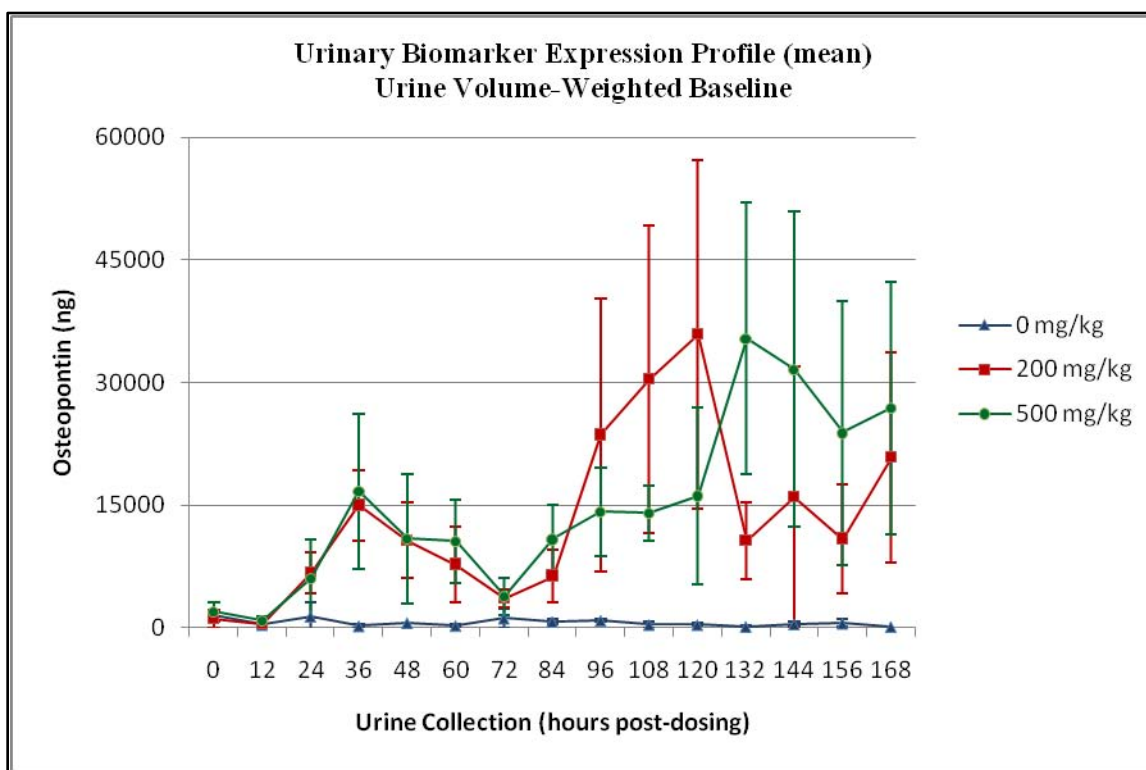
Figure 58: Measurement of urinary OPN (urine volume-weighted) versus time after D-serine administration (Study 2U)
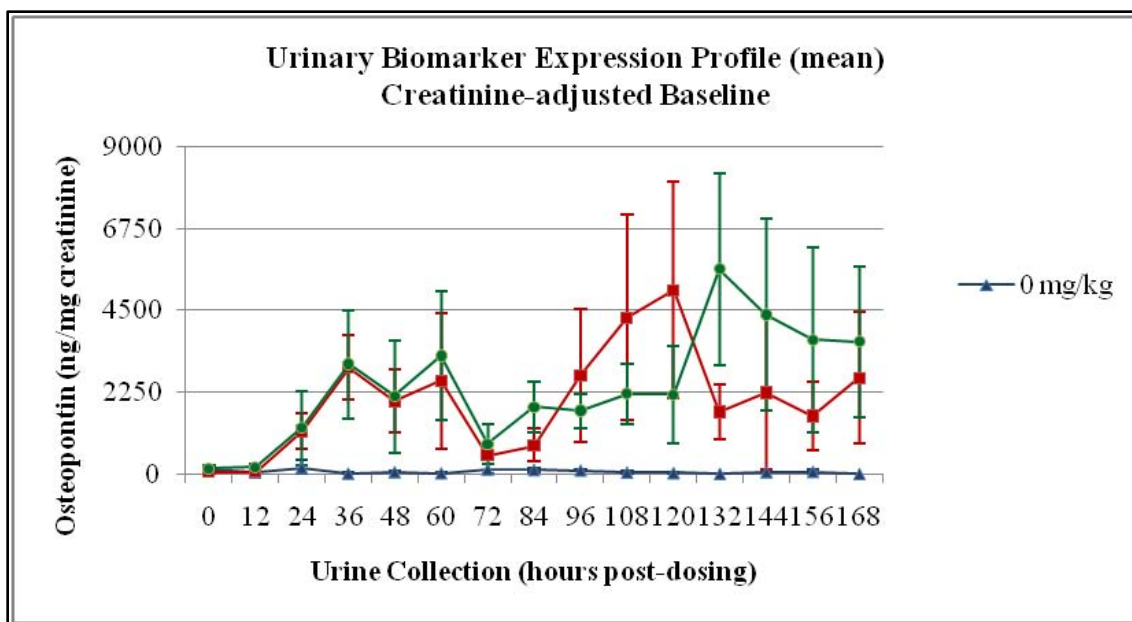
Figure 59: Measurement of urinary OPN (ng/mg creatinine) versus time point after D-serine administration (Study 2U)
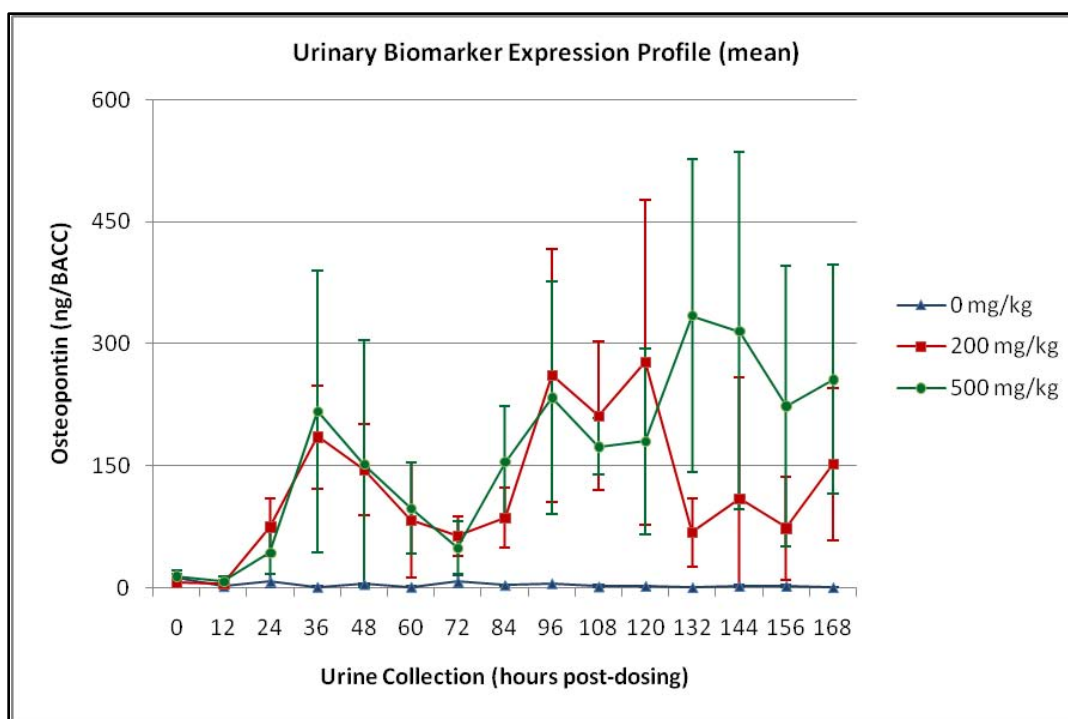
Figure 60: Measurement of urinary OPN (BACC adjusted) versus time point after D-serine administration (Study 2U)

2.10 Kidney Injury Molecule 1 (Kim-1)

2.10.1 Kim-1 Summary

Kim-1 has been noted in many publications as a biomarker to acute, chronic, chemically-induced, or disease-induced kidney damage, and therefore was examined in a nephrotoxin model for its potential use as a marker indicative of toxin-induced renal injury. The protein expression profile of this marker in urine was examined in a time course response study using several doses of the known nephrotoxin D-serine. Male Fischer 344 rats were dosed 200 and 500 mg/kg D-serine in saline, whereas control animals were dosed with saline alone. Urine was collected from animals prior to dosing and at 12 hour intervals in a 168 hour period and frozen immediately. Because no commercial antibodies or ELISA kits were available for detecting rat Kim-1, urine samples were tested for Kim-1 using the MSD Multi-Spot® Assay. Significant increases in the marker were observed with peaks at 96 hours post-dosing and 120 hours post-dosing in dosing groups 200 mg/kg and 500 mg/kg, respectively. Kim-1 expression differed significantly between the control group and dosing group 200 mg/kg and the control group and dosing group 500 mg/kg at time points 96, 108 and 120 hours ($p<0.05$). No statistically significant difference was observed between the 200 mg/kg and 500 mg/kg dosing groups at these time points. Along with the existing journal based data, these expression profiles in response to a kidney toxin indicate that the detection of urinary Kim-1 is a potential noninvasive strategy to predict the early onset of "broad based" kidney damage.

2.10.2 Kim-1 Introduction

Kidney injury molecule 1 (Kim-1) is a 60-100 kDa type 1 membrane glycoprotein and a member of the T cell immunoglobulin and mucin domain-containing (TIM) family of immune regulating adhesion proteins (Rees et al., 2008). Its extra-cellular domain can be shed and serves as an indicator of kidney tubule damage. Kim-1 has been shown to be upregulated in proximal tubule epithelial cells following exposure to various types of nephrotoxicants (Ichimura et al., 2004), suggesting that it may serve as a general biomarker for tubular injury.

Kim-1 has been extensively interrogated as a urinary biomarker to acute kidney injury and, along with clusterin, β-2-microglobulin, NGAL, and to a lesser extent, π and α GST, is considered strong candidates to replace serum creatinine in the clinical laboratory for early diagnosis of kidney injury (Bonventre, 2008; Vaidya et al., 2008). Kim-1 has also been evaluated using various nephrotoxins (S-(1,1,2,2-tetrafluoroethyl)-L-cysteine (TFEC), folic acid, and cisplatin) in a rat model. These data indicate that all three nephrotoxins stimulate Kim-1 expression in the proximal tubule (Ichimura et al., 2004) and this increase can be monitored in the serum and urine.

2.10.3 Kim-1 Methods, Assumptions, and Procedures

Urine and Serum Collection

More detailed descriptions of the animal studies are described in *Biomarkers of Exposure to Toxic Substances, Vol 1* (DelRaso et al., Sep 2009). However, for clarity of this section, we include brief descriptions of each study.

D-serine Study 1

Male Fischer 344 rats were dosed with 5, 20, 50, 200 or 500 mg/kg D-serine in saline, whereas control animals were dosed with saline alone. Five animals were included in each dosing group with the exception of the 200 mg/kg group in which only four animals were included. Urine was collected in metabolic cages from animals prior to dosing and at 24, 48, 72 and 96 hours post-dose . One percent sodium azide, a bacteriostatic preservative, was added to each sample, and urine was frozen at -20°C within 1 hour of end point collection.

D-serine Study 2U(urine)

Male Fischer 344 rats were dosed with 200 or 500 mg/kg D-serine in saline, whereas control animals were dosed with saline alone. Five animals were included in each dosing group with the exception of the control group in which only four animals were included. Urine was collected from animals prior to dosing and at 12-hour intervals up to 168 hours post-dose. Protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) equal to 1/100 of the urine volume was added at collection, and urine was frozen immediately at -20°C before being moved into a -80°C freezer within 1-7 days.

Kidney Injury panel 1 (rat) Multi-Spot® Assay

Multi-Spot® assays were performed as described in the Meso Scale Discovery Kidney Injury Panel 1 (rat) Assay kit protocol. First, urine samples were diluted 1:5 with mouse/rat serum cytokine (MRSC) antibody diluent. Thirty minutes prior to addition to the assay plate, 75 uL of diluted sample were mixed with SULFO-TAG™ albumin tracer solution. A total of 150 uL of blocker A solution were added to the wells of 96-well Multi-Spot® plates coated with capture antibodies for NGAL and Kim-1 and incubated for 1 hour at room temperature with vigorous shaking at approximately 800 rpm. Plates were washed 3 times with phosphate buffered saline plus 0.05% Tween-20 (PBST). Fifty microliters of pre-mixed sample were added to the wells and the plate was incubated for 2 hours at room temperature with vigorous shaking as before, after which the plates were washed three times with PBST. Twenty-five microliters of SULFO-TAG™ kidney injury panel 1 detection antibody blend were added and the plates incubated for 2 hours at room temperature with vigorous shaking as before. Wells were again washed three times with PBST, 150 uL Read buffer T with surfactant was added, and the electrochemiluminescence (ECL) signal measured immediately using a SECTOR® Imager 2400 (Meso Scale Discovery).

Statistical Analysis

All data were the average of duplicate determinations. The results were expressed as mean values ± standard deviations (SD). Statistical methodology included analysis of variance (H0: $\mu_1 = \mu_2 = \ldots = \mu_k$ versus H$_a$: $\mu_i = \mu_j$). When a significant difference was found at $\alpha = .05$, Tukey's multiple comparisons were performed to identify which dosing groups differed significantly. To ensure validity of the analysis of variance, the Kolmogorov-Smirnov test of normality and Bartlett's test for equal variances were performed on the residuals. Where normality was not justified, the Kruskal-Wallis test was performed in lieu of the analysis of

variance, followed by Tukey's multiple comparisons as appropriate. When the assumption of equal variances failed, transformations were performed to stabilize the variance.

2.10.4 Kim-1 Results and Discussions

Kim-1 Multi-Spot® Assay

The MSD Multi-Spot® Kidney Injury Panel 1 (rat) Assay kit was also used to detect Kim-1 in the urine samples of Male Fischer 344 Rats-CDF® (F-344)CrlBR rats dosed with the nephrotoxin D-serine. Because the Kidney Injury Panel 1 Assay is a multiplex assay, by definition it is capable of detecting multiple protein targets simultaneously. The capture antibody against Kim-1 is in the same well as the capture antibody against NGAL, but coated on a distinct electrode. As with detecting NGAL, the Multi-Spot® kit uses a sandwich immunoassay format to detect Kim-1. The LLOD of Kim-1 is 0.0013 ng/mL and the LLOQ 0.02 ng/mL.

Significant biomarker profile changes in Kim-1 were seen in urine in the 200 and 500 mg/kg D-serine dose groups. Kidney histopathology data and clinical chemistry data (TBIL, CREA, BUN, and Total Protein) confirmed sub-clinical kidney damage at the 200 and 500 mg/kg doses (data not shown). A one-way ANOVA indicated that Kim-1 expression differed significantly between the control group and dosing group 200 mg/kg and the control group and dosing group 500 mg/kg at time points 96, 108 and 120 hours ($p < 0.05$). The statistical model required log transformation to pass the assumption of equal variance. No statistically significant difference was observed between the 200 mg/kg and 500 mg/kg dosing groups at the above mentioned time points. The urine profile of the biomarker peaked at 96 hours in dosing group 200 mg/kg, reaching 23 ng/mL, and at 120 hours in dosing group 500 mg/kg, reaching 18 ng/mL (Figure 61).
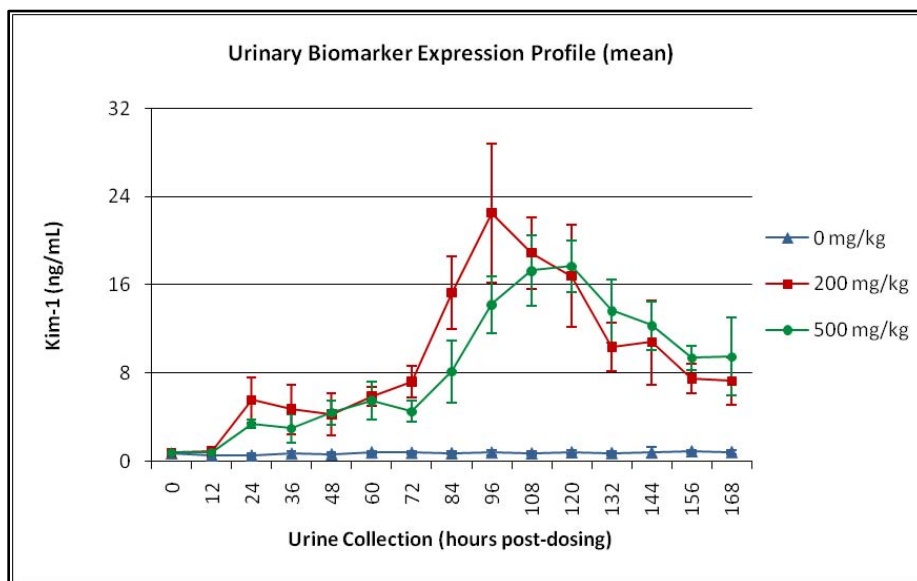
Figure 61: Measurement of urinary Kim-1 by Multi-Spot® Assay verses time after D-serine administration

98

After peak expression, Kim-1 levels in dosing group 200 mg/kg gradually decreased over the next six time points, declining to 7 ng/mL at 168 hours. Kim-1 levels in dosing group 500 mg/kg declined to 10 ng/mL by the final time point. In neither dosing group did Kim-1 levels return to pre-dose concentrations, 0.8 ng/mL.   As discussed previously (Section 2.1), the profile data was normalized and graphed using either urine volume-weighted (Figure 62), creatinine-adjusted (Figure 63), or BACC baseline adjustments (Figure 64) for comparisons to Kim-1 published data.
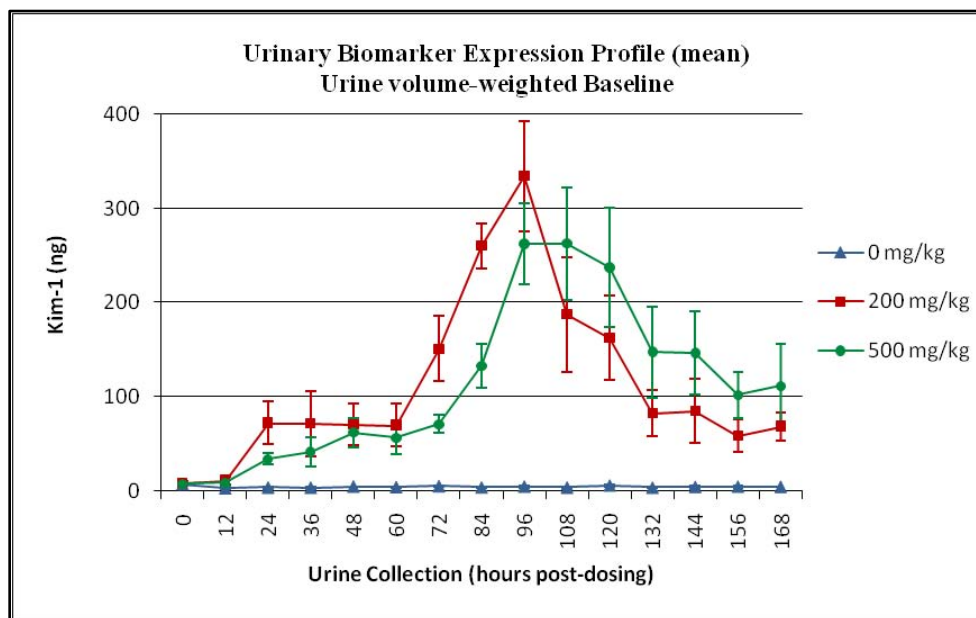
Figure 62: Measurement of urinary Kim-1 (urine volume-weighted) versus time after D-serine administration (Study 2U)
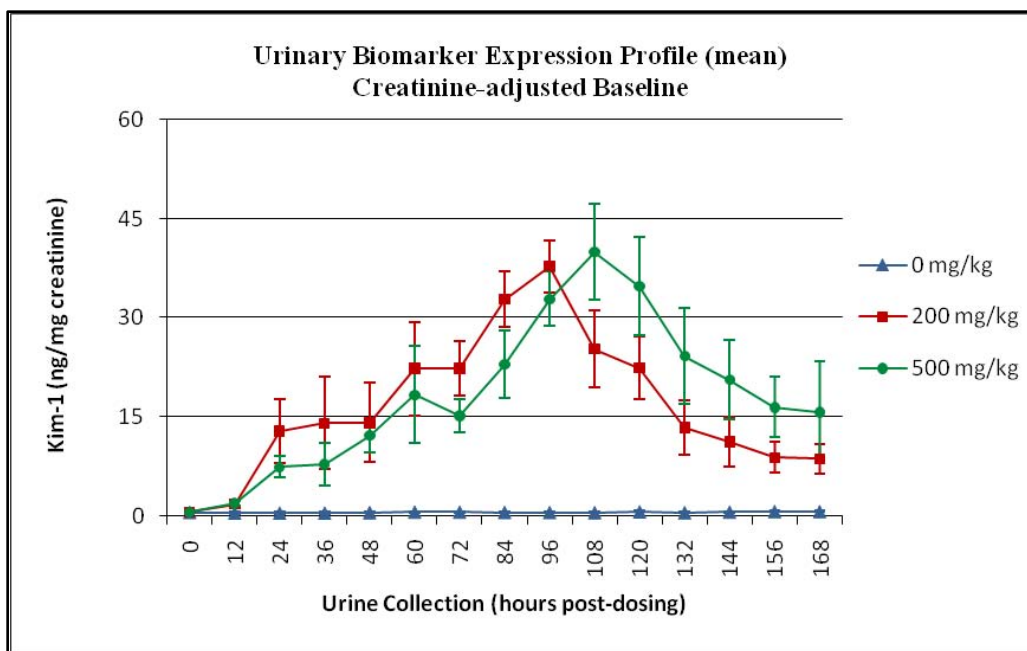
Figure 63: Measurement of urinary Kim-1 (ng/mg creatinine) versus time point after D-serine administration (Study 2U)
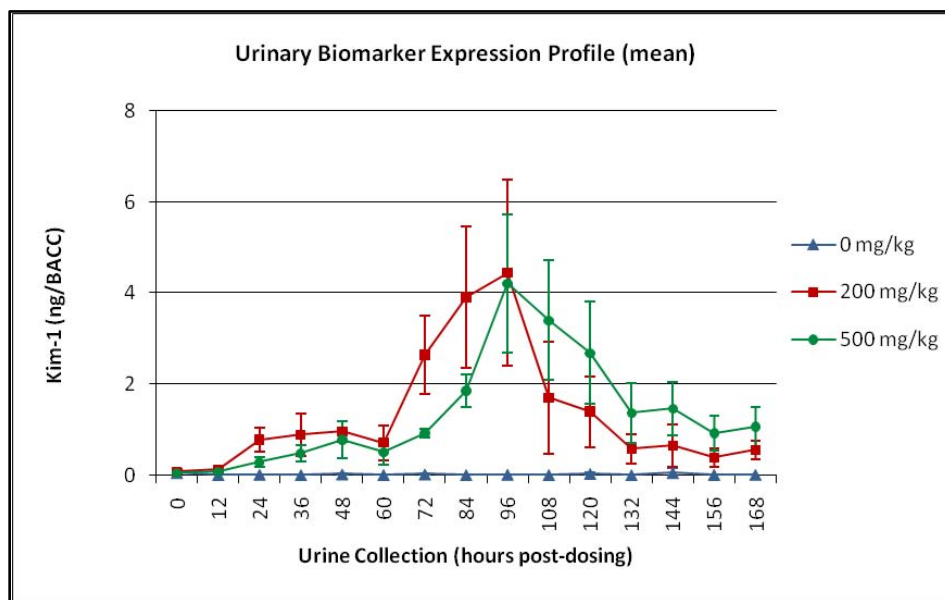
Figure 64: Measurement of urinary Kim-1 (BACC adjusted) versus time point after D-serine administration (Study 2U)

2.11 Neutrophil gelatinase associated lipocalin (NGAL)

2.11.1 NGAL Summary

Neutrophil gelatinase associated liocalin (NGAL) was identified in current literature as a potential biomarker predictive of renal damage. Its urinary expression profile was examined in a time and dose dependent manner using D-serine, a well characterized nephrotoxin affecting the distal tubules of the kidney, which was given at levels previously determined to initiate low-level kidney damage. Male Fischer 344 rats were dosed, and samples collected as previously discussed. The samples were assayed for NGAL by ELISA, and the resultant concentrations fell below the lower limit of quantitation (LLOQ) of the assay. Because the rat NGAL ELISA initially used (BioPorto/Assay Designs) was not sensitive enough to analyze the protein in our model system and test matrix, a Meso Scale Discovery (MSD) Multi-Spot® Assay was used as an alternative and presumably more sensitive method However, as was observed with the BioPorto ELISA, biomarker levels fell below the LLOQ using the Multi-Spot® Assay to test for NGAL in urine. However, the majority of samples did fall within the detection range of the assay. Based on results calculated below the LLOQ but above the lower limit of detection (LLOD), NGAL expression peaked at 96 hours post-dosing in the 200 mg/kg dosing group and at 120 hours post-dosing in the 500 mg/kg dosing group. At these time points, NGAL expression was significantly higher in the dosing groups than the control group and dosing group 200 mg/kg was significantly higher than dosing group 500 mg/kg ($p<0.05$). It was interesting that eh NGAL levels fell below the LLOQ in both assays – perhaps NGAL is not released at levels seen in other publications due to the low level of renal damage initiated in this model. Along with the extensive published data on NGAL, these expression profiles indicate that the detection of urinary NGAL may be a viable biomarker indicative of very early kidney tubule damage with the development of more sensitive NGAL assays.

2.11.2 NGAL Introduction

Rat NGAL is a 20.5 kDa peptide chain composed of 178 amino-acid residues and belongs to the lipocalin super family (Kjeldsen et al., 2000). While initially found in activated neutrophils, it has been shown that in the adult rat, the protein is expressed in the liver, prostate, kidney, mammary gland and epithelial cells of the respiratory and alimentary tracts. Many types of cells, including in the kidney tubule, produce NGAL in response to injury (Bolignano et al., 2008). In nephrotoxin-induced acute renal failure of humans, NGAL protein accumulates in the blood, urine, renal proximal tubules and renal distal tubules (Mori et al., 2007). NGALs small molecular size and protease resistance may render it readily detectable in urine (Ding et al., 2007).

2.11.3 NGAL Methods, Assumptions, and Procedures

Urine and Serum Collection

More detailed descriptions of the animal studies are described in *Biomarkers of Exposure to Toxic Substances, Vol 1* (DelRaso et al., Sep 2009). However, for clarity of this section, we include brief descriptions of each study.

D-serine Study 1

Male Fischer 344 rats were dosed with 5, 20, 50, 200 or 500 mg/kg D-serine in saline, whereas control animals were dosed with saline alone. Five animals were included in each dosing group with the exception of the 200 mg/kg group in which only four animals were included. Urine was collected in metabolic cages from animals prior to dosing and at 24, 48, 72 and 96 hours post-dose . One percent sodium azide, a bacteriostatic preservative, was added to each sample, and urine was frozen at -20°C within 1 hour of end point collection.

D-serine Study 2U(urine)

Male Fischer 344 rats were dosed with 200 or 500 mg/kg D-serine in saline, whereas control animals were dosed with saline alone. Five animals were included in each dosing group with the exception of the control group in which only four animals were included. Urine was collected from animals prior to dosing and at 12-hour intervals up to 168 hours post-dose. Protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) equal to 1/100 of the urine volume was added immediately after collection, and urine frozen immediately at -20°C. Samples were moved into a -80°C freezer within 1-7 days for long term storage.

D-serine Study 2S(serum)

Male Fischer 344 rats were dosed with 200 or 500 mg/kg D-serine in saline, whereas control animals were dosed with saline alone. Ten animals were included in each dosing group. Five of the ten animals were sacrificed 12 hours post-dose. The remaining five animals were sacrificed 24 hours post-dose. Blood was drawn via the inferior vena cava using a 10 cc syringe fitted with an 18 G needle, placed in a 10 mL vacutainer (no additives), and allowed to sit on ice for 20 minutes. The serum was collected off the top of the tube, placed in cryovials, and frozen in liquid nitrogen.

NGAL ELISA

Enzyme-linked immunosorbent assays (ELISA) were performed as described in the BioPorto® rat NGAL ELISA kit protocol. First, urine samples were diluted 1:15 with sample diluent. One hundred microliters of diluted sample were added to the wells of 96-well microtiter plates coated with a monoclonal antibody against rat NGAL and incubated for 1 hour at room temperature with uniform shaking. Plates were washed 3 times with wash solution. One hundred microliters of biotinylated rat NGAL antibody were added and incubated for 1 hour at room temperature with uniform shaking. Wells were washed three times, 100 uL of HRP-streptavidin solution was added to the wells, and plates were incubated for 1 hour at room temperature with uniform shaking. Wells were washed three times, 100 uL of TMB substrate (3,3',5,5'-tetramethylbenzidine) was added, and plates were incubated exactly 10 minutes at room temperature in the dark. Reactions were stopped by the addition of 100 uL of 0.5 M sulfuric acid. The absorbance (optical density [OD]) was measured at 450 nm (reference 650 nm) with a microplate reader (Molecular Devices SpectraMax M2^e).

Kidney Injury panel 1 (rat) Multi-Spot® Assay

Multi-Spot® assays were performed as described in the Meso Scale Discovery Kidney Injury Panel 1 (rat) Assay kit protocol. First, urine samples were diluted 1:5 with mouse/rat serum cytokine (MRSC) antibody diluent. Thirty minutes prior to addition to the assay plate, 75 uL of diluted sample were mixed with SULFO-TAG™ albumin tracer solution. A total of 150 uL of blocker A solution were added to the wells of 96-well Multi-Spot® plates coated with capture antibodies for NGAL and Kim-1 and incubated for 1 hour at room temperature with vigorous shaking at approximately 800 rpm. Plates were washed 3 times with phosphate buffered saline plus 0.05% Tween-20 (PBST). Fifty microliters of pre-mixed sample were added to the wells of the plate and incubated for 2 hours at room temperature with vigorous shaking at approximately 800 rpm. Plates were washed three times with PBST. Twenty-five microliters of SULFO-TAG™ kidney injury panel 1 detection antibody blend were added and incubated for 2 hours at room temperature with vigorous shaking at approximately 800 rpm. Wells were washed three times with PBST and 150 uL of Read buffer T with surfactant were added, and the electrochemiluminescence (ECL) signal was measured immediately using a SECTOR® Imager 2400 (Meso Scale Discovery).

Statistical Analysis

All data were the average of duplicate determinations. The results were expressed as mean values ± standard deviations (SD). Statistical methodology included analysis of variance (H0: $\mu_1 = \mu_2 = \ldots = \mu_k$ versus H_a: $\mu_i = \mu_j$). When a significant difference was found at $\alpha = .05$, Tukey's multiple comparisons were performed to identify which dosing groups differed significantly. To ensure validity of the analysis of variance, the Kolmogorov-Smirnov test of normality and Bartlett's test for equal variances were performed on the residuals. Where normality was not justified, the Kruskal-Wallis test was performed in lieu of the analysis of variance, followed by Tukey's multiple comparisons as appropriate. When the assumption of equal variances failed, transformations were performed to stabilize the variance.

2.11.4 NGAL Results and Discussions

NGAL ELISA

The known nephrotoxin D-serine was used to induce sub-clinical kidney damage at the proximal straight tubules in order to evaluate whether NGAL could serve as an early predictor of nephrotoxicity. Doses of 200 or 500 mg/kg D-serine were administered intraperitoneally in 0.9% saline, while control animals were dosed with saline alone. Urine samples were collected at 12 hour intervals for a total of 168 hours. NGAL was then quantitated by sandwich ELISA and the biomarker expression profile was determined.

Because absolute concentrations of NGAL in rat urine are unknown, and immunoassay results have not been standardized to an accepted purified preparation of the protein, ELISA results were recorded as units/mL. The lower limit of detection (LLOD) of the BioPorto® NGAL ELISA assay was 0.009 units/mL. While the lower limit of quantitation (LLOQ) was not defined in the kit insert, BioPorto and Assay Designs, Inc., distributor of the kit, stated in an e-

mail communication that the LLOQ is equal to the lowest calibrator, 0.05 units/mL, and that results should not be extrapolated past the range of the calibrators.

Based on an LLOQ equal to the lowest calibrator, all results, with the exception of one sample (337-0mg/kg-12h), fell below the LLOQ. Therefore, the BioPorto® rat NGAL ELISA kit is not sensitive enough to quantitate the protein in Male Fischer 344 Rats-CDF® (F-344) CrlBR.

NGAL Multi-Spot® Assay

The Meso Scale Discovery (MSD)Multi-Spot® Kidney Injury Panel 1 (rat) Assay kit was used as an alternative method to detect NGAL in rat urine samples. As with the NGAL ELISA, the Multi-Spot® kit uses a sandwich immunoassay format to detect NGAL. Unlike in a colorimetric ELISA, however, the detection antibody in the Multi-Spot® Assay is labeled with an electrochemiluminescent MSD SULFO-TAG™ label. A SECTOR® instrument is used to apply a voltage to electrodes coated on the plates causing the SULFO-TAG™ to emit light. The intensity of the emitted light is used to provide a quantitative measure of NGAL. According to the kit insert, the LLOD of NGAL in the Multi-Spot® Kidney Injury Panel 1 (rat) Assay is 0.250 ng/mL, or 2.5 standard deviations above the background signal. The LLOQ is 1.56 ng/mL, or the lowest concentration where the percent CV (coefficient of variation) of the calculated concentration is less than 20% and the percent recovery of the standard is between 80% and 120%.

Based on the limits of detection and quantitation established by MSD and taking into consideration the dilution of the samples, all samples fell below the LLOQ of the assay. As was observed with the BioPorto® rat NGAL ELISA kit, the MSD Multi-Spot® Assay was not sensitive enough to quantitate NGAL in the nephrotoxin urine sample set. However, while all samples fell below the LLOQ of the Multi-Spot® Assay, 188 out of the 210 samples tested did fall within the detection range of the assay. For the sake of visualizing a trend in the urinary expression of NGAL, the collected data was graphed (Figure 65). Concentrations were extrapolated for samples that were below the LLOQ but fell within the detection range. Samples that fell below the detection range were recorded as 0 ng/ml. As the samples fell below the LLOQ, no base-line adjustment graphs of the data (BACC, urine or creatinine weighted) were accomplished.
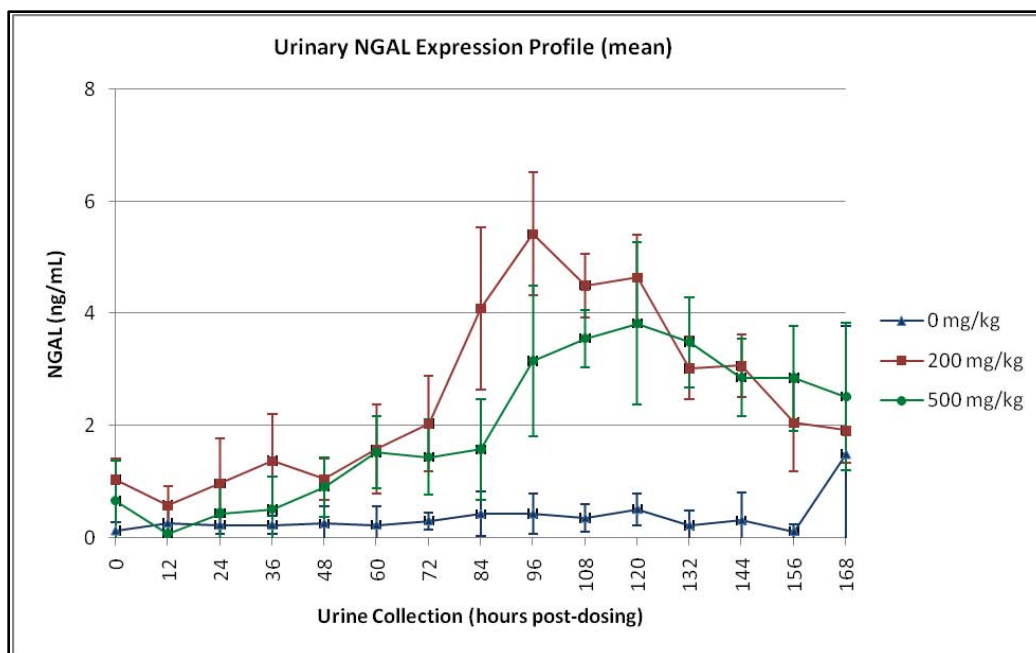
Figure 65: Measurement of urinary NGAL by Multi-Spot®; Assay over time after D-serine administration
Note: The data presented in this graph are based on extrapolations from a standard curve at concentrations below the LLOQ determined by the manufacturer of the kit

A one-way analysis of variance (ANOVA) showed that NGAL expression differed significantly between dosing groups 0, 200 and 500 mg/kg at time points 96, 108 and 120 hours. The control group was significantly lower than the 200 mg/kg and 500 mg/kg dosing groups, and dosing group 200 mg/kg was significantly higher than dosing group 500 mg/kg ($p<0.05$). Kidney histopathology data and clinical chemistry data (TBIL, CREA, BUN, and Total Protein) confirmed sub-clinical kidney damage at the 200 and 500 mg/kg doses (data not shown). While siginificant uring NGAL response has been seen in other studies (Mori et al., 2007), the fact that the NGAL concentrations fell belowe the LLOQ may indicate that this marker may not be suitable for the detection of subclinical levels of renal damage. NGAL may be viable for use with toxin-based renal damage, however, with the development of more sensitive NGAL assays.

2.12 Renal Papillary Antigen 1 (RPA-1)

2.12.1 RPA-1 Summary

Renal Papillary Antigen-1 (RPA-1) is a biomarker which has been shown to be predictive of chemically-induced and disease-induced kidney damage. The protein expression profile of RPA-1 in urine was examined in a time course response using several doses of the known nephrotoxin D-serine. Male Fischer 344 rats were dosed with 5, 20, 50, 200, or 500 mg/kg D-serine in saline, whereas control animals were dosed with saline alone. Urine was collected from animals prior to dosing and at 24, 48, 72 and 96 hours post-dosing and frozen within 1 hour of end point collection. The samples were assayed by ELISA for renal papillary antigen-1 (RPA-1) using a Biotrin rat RPA-1 Enzyme Immunoassay kit. Biomarker levels remained constant for control animals and in animals dosed with 5, 20, and 50 mg/kg D-serine throughout the time course. However, for animals dosed with 200 and 500 mg/kg D-serine, significant increases were observed with peaks at 48 hours post-dosing. RPA-1 levels dropped at 72 hours post-dosing. However, marked increases in OPN and RPA-1 levels were again observed at 96 hours post-dosing. A second time-course experiment was conducted in order to confirm the peak response times of each biomarker. Again, D-serine was used to induce sub-clinical kidney damage at the proximal straight tubules. Doses of 200 or 500 mg/kg D-serine were administered intraperitoneally in 0.9% saline. Control animals were dosed with saline alone. Urine samples were collected at 12 hour intervals in a 168 hour period and frozen immediately. The potential biomarkers were quantitated by sandwich ELISA Urine samples from the second time course experiment were not assayed for RPA-1 because the protein was excluded as a suitable biomarker. Our studies indicate that RPA-1 does not seem to be responsive to low levels of proximal tubule damage in response to D-serine nephrotoxicity.

2.12.2 RPA-1 Introduction

Study examining increased expression of RPA-1, RAP-2, and Kim-1 in gentamicin sulfate and cisplatin treated rat kidneys indicate that RPA-1 increased in the epithelial cells of the collecting ducts of the cortex (gentamicin treated) or in the epithelial cells of the medullary collecting duct (cisplatin treated) (Zhang et al., 2009). Examination of damage to rat renal papilla (Falkenberg et al., 1996) demonstrated that RPA-1 is released into the urine of injury to collecting duct or papillary injury.

2.12.3 RPA-1 Methods, Assumptions, and Procedures

Urine and Serum Collection

More detailed descriptions of the animal studies are described in *Biomarkers of Exposure to Toxic Substances, Vol 1* (DelRaso et al., Sep 2009). However, for clarity of this section, we include brief descriptions of each study.

D-serine Study 1

Male Fischer 344 rats were dosed with 5, 20, 50, 200 or 500 mg/kg D-serine in saline, whereas control animals were dosed with saline alone. Five animals were included in each

dosing group with the exception of the 200 mg/kg group in which only four animals were included. Urine was collected in metabolic cages from animals prior to dosing and at 24, 48, 72 and 96 hours post-dose . One percent sodium azide, a bacteriostatic preservative, was added to each sample, and urine was frozen at -20°C within 1 hour of end point collection.

D-serine Study 2U(urine)

Male Fischer 344 rats were dosed with 200 or 500 mg/kg D-serine in saline, whereas control animals were dosed with saline alone. Five animals were included in each dosing group with the exception of the control group in which only four animals were included. Urine was collected from animals prior to dosing and at 12-hour intervals up to 168 hours post-dose. Protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) equal to 1/100 of the urine volume was added, and urine was frozen immediately at -20°C before being moved into a -80°C freezer within 1-7 days.

D-serine Study 2S(serum)

Male Fischer 344 rats were dosed with 200 or 500 mg/kg D-serine in saline, whereas control animals were dosed with saline alone. Ten animals were included in each dosing group. Five of the ten animals were sacrificed 12 hours post-dose. The remaining five animals were sacrificed 24 hours post-dose. Blood was drawn via the inferior vena cava using a 10 cc syringe fitted with an 18 G needle, placed in a 10 mL vacutainer (no additives), and allowed to sit on ice for 20 mintues. After incubation, the tubes were centrifuged at 2500 x g for 10 minutes. The serum was collected off the top of the tube, placed in cryovials, and frozen in liquid nitrogen.

Renal Papillary Antigen 1 ELISA

ELISAs were performed as described in the Biotrin rat RPA-1 Enzyme Immunoassay kit protocol. First, urine samples were diluted 1:25 with sample diluent. One hundred microliters of diluted sample were added to the wells of 96-well microtiter plates coated with IgG directed against rat renal papillary antigen-1 (RPA-1) and incubated for 1 hour at 20-25°C with uniform shaking. Plates were washed six times with wash solution (tris-buffered saline containing Tween 20). One hundred microliters of enzyme conjugate (anti-rat RPA-1 IgG conjugated to horseradish peroxidase) were added and incubated for 1 hour at 20-25°C with uniform shaking. Wells were washed six times, 100 uL of substrate solution (stabilized tetramethylbenzidine solution) was added to the wells, and plates were incubated for 15 minutes at 20-25°C. Reactions were stopped by the addition of 100 uL of 1 N H_2SO_4. The absorbance (optical density [OD]) was measured at 450-630 nm with a microplate reader (Molecular Devices SpectraMax M2^e).

Statistical Analysis

All data were the average of duplicate determinations. The results were expressed as mean values ± standard deviations (SD). Statistical methodology included analysis of variance (H0: $\mu_1 = \mu_2 = \ldots = \mu_k$ versus H$_a$: $\mu_i = \mu_j$). When a significant difference was found at $\alpha = .05$, Tukey's multiple comparisons were performed to identify which dosing groups differed

significantly. To ensure validity of the analysis of variance, the Kolmogorov-Smirnov test of normality and Bartlett's test for equal variances were performed on the residuals. Where normality was not justified, the Kruskal-Wallis test was performed in lieu of the analysis of variance, followed by Tukey's multiple comparisons as appropriate. When the assumption of equal variances failed, transformations were performed to stabilize the variance.

2.12.4 RPA-1 Results and Discussions

D-Serine Study 1 Urine Analysis

The known nephrotoxin D-serine was used to induce sub-clinical kidney damage at the proximal straight tubules in order to evaluate whether RPA-1 could serve as an early predictor of nephrotoxicity. Doses of 20, 50, 200 or 500 mg/kg D-serine were administered intraperitoneally in 0.9% saline. Control animals were dosed with saline alone. Urine samples were collected at 24 hour intervals for a total of 96 hours. RPA-1 was quantitated via sandwich ELISA and the biomarker expression profile was determined (Figure 66).
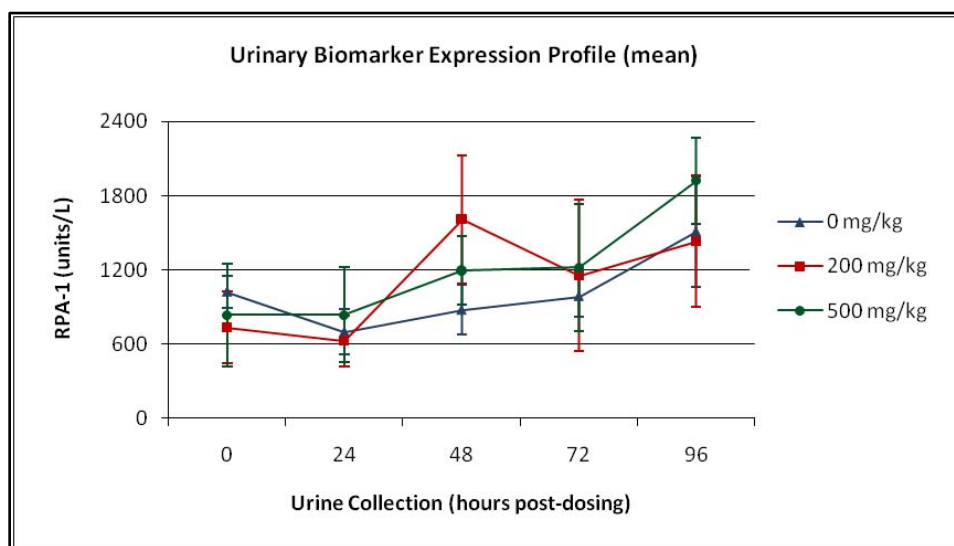
Figure 66: Measurement of urinary RPA-1 by ELISA versus time after D-serine administration

No significant biomarker profile changes in RPA-1 were observed at the 5, 20, 50, 200 and 500 mg/kg D-serine dose groups, despite kidney histopathology data and clinical chemistry data (TBIL, CREA, BUN, Total Protein) indicating the initiation of kidney damage around the 200 mg/kg dose (data not shown). One-way ANOVA indicated no significant differences between dosing groups 0, 200 and 500 mg/kg. A one-way ANOVA with multiple comparisons was performed between all five time points in dosing group 0 mg/kg. RPA-1 expression at time point 96 hours reached 1509 units/mL and differed significantly from concentrations at all other time points. No significant differences were observed between RPA-1 concentrations at 0, 24, 48 or 72 hours. A one-way ANOVA followed by multiple comparisons using $\alpha = 0.10$ was performed between all five time points in dosing group 200 mg/kg. The highest concentration of

urinary RPA-1 was observed at time point 48 hours. RPA-1 levels reached 1610 units/mL, which differed significantly from the 625 units/mL observed at 24 hours. No significant differences were observed between RPA-1 concentrations at any other pairs of time points. A one-way ANOVA with multiple comparisons was performed between all five time points in dosing group 500 mg/kg, where RPA-1 expression peaked at 96 hours at 1921 units/mL. The only statistically significant differences between the concentrations among the time points of this dose group were between 96 and 0 hours and 96 and 24 hours. In conclusion, while some statistically significant changes can be seen within the post-dose period in a given dose, no significant changes between the control and dosed groups were detected. Thus, for the detection of low-level renal damage, at least induced at the proximal tubule, urinary RPA-1 is not a viable marker.

2.13 Endothelin-1 (ET-1)

2.13.1 Endothelin-1 Summary

Published data indicated that endothelin-1 (ET-1) levels were elevated in several kidney injury models, identifying this protein as a potential biomarker of kidney injury. Therefore, endothelin-1 was included as a possible biomarker to be examined in a D-serine rat study modeling subclinical renal damage. To initiate low level kidney damage at the kidney tubule or glomerulus, Male Fischer 344 rats were dosed with 0, 5, 20, 50, 200, or 500 mg/kg D-serine in saline or 0, 5, 25, 75, or 150 mg/kg puromycin in saline. Urine samples were kept cold during sampling intervals of 24, 48, 72 and 96 hours post-dose, and frozen within 1 hour of collection. Histopathological examination of dosed kidney tissue verified the levels of damage at each dose. In order to quantitate endothelin-1 in urine, a commercially available ELISA kit from ALPCO Diagnostics was evaluated for its ability to detect endothelin in rat urine samples. The ALPCO endothelin ELISA kit is validated by the company for use with human serum, EDTA plasma, urine, saliva and cell culture, but not in rat urine. We examined the ability of this assay to detect endothelin-1 in rat urine samples spiked with known levels of the protein, and determined that dilutional linearity could not be produced in our test matrix with this kit. As the kit failed validation in rat urine, we did not fully examine the expression of ET-1 in the D-serine nephrotoxin rat model. It is thought that the human-based antibodies in the ALPCO kit did not have sufficient cross reactivity to quantitatively bind rat endothelin-1. As the kit was validated for use in urine, salt interference was not thought to be an issue. While ET-1 may indeed prove to be an adequate biomarker to toxin-induced kidney damage, further studies using a rat-specific ET-1 ELISA will be needed.

2.13.2 Endothelin-1 Introduction

Endothelin-1 (ET-1) is a small 21 amino acid peptide which exhibits vasoconstriction properties (Yanagisawa et al., 1988) under profibrotic and pro-inflammatory conditions. ET-1 is produced in several types of cells, including epithelial cells of the kidney (Richter, 2006; Neuhofer et al., 2006). Research indicates that ET-1 is involved in moderating normal function of the kidney, primarily the glomerular filtration rate and reabsorption of both solutes and water. More recent research indicate that these peptides are also produced in response to a wide range of renal conditions, such as post-ischemic acute renal failure (Mitterbauer et al., 2003), acute renal failure (Li et al., 1999, Yanagisawa et al., 1998), and diabetes (Kalani, 2008). The extensive role of endothelins in the normal renal function as well as in the diseased organ is reviewed in Kohan (1997). ET-1 activity was identified in the urine, and ET-1 levels were shown to increase in the urine in patients with chronic renal failure (Fujisaki et al., 2003) leading to the possibility of its utility as a urinary biomarker indicative of kidney injury.

2.13.3 Endothelin-1 Methods, Assumptions, and Procedures

Urine Collection D-serine Study 1

Male Fischer 344 rats were dosed with 5, 20, 50, 200 or 500 mg/kg D-serine in saline, whereas control animals were dosed with saline alone. Five animals were included in each dosing group with the exception of the 200 mg/kg group in which only four animals were included. Urine was collected in metabolic cages from animals prior to dosing and at 24, 48, 72 and 96 hours post-dosing . One percent sodium azide, a bacteriostatic preservative, was added to each sample, and urine was frozen at -20°C within 1 hour of end point collection.

Endothelin-1(1-21) ELISA

Enzyme-linked immunosorbent assays were performed as described in the ALPCO Diagnostics Endothelin-1 (1-21) kit protocol (ALPCO Diagnostics, NH). The ALPCO ELISA used is validated for detection of endothelin-1 in human serum, plasma, urine, saliva and cell culture supernatants, with 79% cross-reactivity in rat sera. It had not been validated for use with rat urine. To initiate validation tests, standards and quality controls were prepared in distilled water. Urine samples were diluted with 0.9% NaCl. Fifty microliters of diluted sample were added to the wells of 96-well microtiter plates coated with polyclonal anti-endothelin-1 antibody. Two hundred microliters of monoclonal mouse anti-endothelin-1 detection antibody were added to each well, and the plates were incubated overnight at room temperature (18-26 °C). Plates were washed 5 times with wash buffer. Two hundred microliters of anti-mouse IgG antibody-HRPO were added and incubated for 1 hour at room temperature. Wells were washed five times, 200 uL of TMB substrate was added to the wells, and plates were incubated for 30 minutes at room temperature in the dark. Reactions were stopped by the addition of 50 uL of 2 M sulfuric acid. The absorbance (optical density [OD]) was measured at 450-620 nm with a microplate reader (Molecular Devices SpectraMax M2[e]).

Endothelin-1 (1-21) ELISA Kit Evaluation 1

For one urine sample expected to have measurable levels of endothelin-1 (500 mg/kg dose, 24 hour post-dose), an 8-point, 2-fold serial dilution was performed, beginning at 1:2 and ending at 1:256. Reference standard material at concentrations of 0 and 2 fmol/mL of endothelin-1 were spiked into a baseline urine sample diluted 1:2, 1:20 and 1:200. All samples were diluted in 0.9% NaCl.

Endothelin-1 (1-21) ELISA Kit Evaluation 2

For one urine sample expected to have measurable levels of endothelin-1, an 8-point, 2-fold serial dilution was performed, beginning at 1:4 and ending at 1:512. Reference standard material at concentrations of 0 and 2 fmol/mL of endothelin-1 were spiked into a baseline urine sample diluted 1:4, 1:40 and 1:400. All samples were diluted in distilled water.

2.13.4 Endothelin-1 Results and Discussion

Endothelin (1-21) ELISA Kit Evaluation

The ALPCO Diagnostic Endothelin (1-21) ELISA kit is validated for the quantitative determination of human ET-1 in serum, EDTA plasma, urine, saliva and cell culture supernatants, but not in biological matrices from rat. Therefore, the kit was evaluated by three independent analyses in order to assess the kit's capability of detecting ET-1 in rat urine. First, the recovery efficiency of ET-1 from the urine matrix was assessed through a spike and recovery experiment. Second, the kit's capability of detecting ET-1 from in-house rat urine samples expected to have measurable levels of the protein was assessed. Third, the linearity of the results obtained from multiple dilutions of a urine sample was assessed.

Endothelin (1-21) ELISA Kit Evaluation 1

The kit insert did not recommend a starting dilution of samples. Therefore, the first evaluation was designed to assay a urine sample expected to have relatively high levels of ET-1 at multiple dilutions to establish the appropriate sample dilution. Additionally, a spike and recovery experiment was performed to assess the recovery efficiency of ET-1 from the urine matrix. Various dilutions of a baseline urine sample were prepared in 0.9% NaCl. The urine dilutions covered the range of dilutions anticipated to be used in regular sample testing. The standard material was then spiked into the urine dilutions and recovery was assessed.
For all eight dilutions of the urine sample, the ODs fell within the range of the standard. However, no dilutional linearity was observed. Rather than seeing a decrease in ODs as the dilution factor increased, a slight increase in ODs was observed (Table 20). Consequently, the calculated protein concentration also increased slightly (Table 20, red text). This increase became amplified when taking into account the dilution factor. It was speculated that the NaCl in the diluent is generating background signal and may also be interfering with the binding of any ET-1 that may be present in the test sample.

Table 20: Analysis of rat urine at dilution factors of 2, 4, 8, 16, 32, 64, 128 and 256

Replicate OD Values	Result (fmol/mL)	**Mean Result**	SD	% CV	Dilution Factor	Adjusted Result (fmol/mL)
0.234	0.189	**0.144**	0.064	44.2	2	0.287
0.210	0.099					
0.219	0.132	**0.183**	0.072	39.3	4	0.733
0.247	0.234					
0.231	0.176	**0.218**	0.059	27.2	8	1.740
0.254	0.259					
0.288	0.380	**0.363**	0.023	6.4	16	5.815
0.278	0.347					
0.343	0.573	**0.492**	0.115	23.3	32	15.744
0.296	0.411					
0.280	0.353	**0.348**	0.007	2.1	64	22.261
0.277	0.343					
0.258	0.273	**0.329**	0.080	24.2	128	42.147
0.289	0.386					
0.332	0.537	**0.521**	0.022	4.3	256	133.350
0.323	0.505					

At first glance, recovery of 2 fmol/mL of ET-1 from urine diluted 1:2, 1:20 and 1:200 was approximately 100% (Table 21, yellow highlights). However, measureable levels of endothelin-1 were detected in the urine sample not spiked with the protein. Taking into account the baseline level of endothelin-1 observed in the 0 fmol/mL spiked sample, the percent recovery of the protein in the 2 fmol/mL spiked sample was only 50%. In addition, dilutional linearity was not observed between the non-spiked urine diluted 1:2, 1:20 and 1:200.

Table 21: Spike and recovery of 0 and 2 fmol/mL endothelin-1
Endothelin-1 (reference standard material) aliquoted into 1:2, 1:20 and 1:200 dilutions of baseline urine sample

Spiked Concentration (fmol/mL)	Mean Adjusted Result (fmol/mL)	Replicate % CV	Dilution Factor	Intra-Assay Mean Adjusted Result (fmol/mL)	Intra-Assay % CV	Percent Recovery
0	1.386	15.4	2	1.010	32.4	NA
	0.858	31.0	20			
	0.787	56.4	200			
2	2.023	11.4	2	2.009	0.7	50
	2.009	8.2	20			
	1.995	21.5	200			

The dilution experiment demonstrated no dilutional linearity between samples diluted 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128 and 1:256. In addition, no dilutional linearity was observed between the non-spiked urine diluted 1:2, 1:20 and 1:200 during the spike and recover experiment. The recovery of endothelin-1 from the 2 fmol/mL spike urine sample was only 50%. Since it was speculated that the NaCl in the diluent is generating background signal and may also be interfering with the binding of any endothelin-1 that may be present in the test sample, both experiments were repeated using distilled water as the sample diluent.

Endothelin (1-21) ELISA Kit Evaluation 2

As previously observed, no dilutional linearity was observed for the eight dilutions of the urine sample (Table 22). Despite using distilled water as the diluent, only background noise was detected.

Table 22: **Endothelin analysis of rat urine at dilution factors of 4, 8, 16, 32, 64, 128, 256 and 512**

Replicate OD Values	Result (fmol/mL)	Mean Result (fmol/mL)	SD	% CV	Dilution Factor	Adjusted Result (fmol/mL)
0.448	0.946	0.993	0.067	6.7	4	3.974
0.472	1.041					
0.528	1.263	1.271	0.011	0.9	8	10.165
0.532	1.279					
0.343	0.517	0.877	0.509	58.0	16	14.040
0.522	1.238					
0.326	0.447	0.480	0.048	9.9	32	15.372
0.342	0.514					
0.292	0.301	0.393	0.131	33.3	64	25.156
0.335	0.486					
0.291	0.297	0.285	0.016	5.7	128	36.495
0.286	0.274					
0.307	0.365	0.522	0.222	42.5	256	133.659
0.382	0.679					
0.280	0.245	0.365	0.169	46.2	512	186.676
0.335	0.484					

As previously observed, measureable levels of ET-1 were detected in the urine sample not spiked with the protein (Table 23). Taking into account the baseline level of endothelin-1 observed in the 0 fmol/mL spiked sample, the percent recovery of the protein in the 2 fmol/mL spiked sample was only 64%. In addition, dilutional linearity was not observed between the non-spiked urine diluted 1:4, 1:40 and 1:400.

Table 23: **Spike and recovery of 0 and 2 fmol/mL Endothelin-1**

Endothelin-1 (reference standard material) aliquoted into 1:4, 1:40 and 1:400 dilutions of baseline urine sample

Spiked Concentration (fmol/mL)	Mean Adjusted Result (fmol/mL)	Replicate % CV	Dilution Factor	Intra-Assay Mean Adjusted Result (fmol/mL)	Intra-Assay % CV	Percent Recovery
0	1.438	11.7	4	0.718	86.8	NA
	0.365	79.1	40			
	0.352	33.5	400			
2	2.170	9.4	4	2.010	8.6	64.3
	2.034	6.3	40			
	1.826	18.0	400			

The results from the kit evaluation indicated that ET-1 is not detected in the rat urine samples. Since the kit was developed to detect human ET-1, it is likely that the human antibody is not cross-reactive with the rat protein. It is also possible that urinary rat ET-1 is more susceptible to proteolysis and thus is degrading during the required overnight room temperature incubation. The Endothelin (1-21) ELISA kit from ALPCO was not included in the study.

2.14 Interleukin 18 (IL-18)

2.14.1 Interleukin-18 Summary

Numerous studies examining Interleukin-18 (IL-18) levels in serum and urine indicate that IL-18 levels are elevated in several kidney injury models, thus identifying this protein as a potential biomarker of kidney injury. Therefore, IL-18 was included as a possible biomarker to examine in urine samples from a D-serine study modeling subclinical kidney damage in the rat. To model low level kidney injury, Male Fischer 344 rats were dosed with 0, 5, 20, 50, 200, or 500 mg/kg D-serine in saline. Urine samples were kept cold during collection periods of 24, 48, 72 and 96 hours post-dose, and frozen within 1 hour at each time point. Renal tissue damage was verified by histopathological examination of control and dosed kidney tissue. In order to quantitate the IL-18 protein in rat urine samples, a commercially available ELISA kit from BioSource was evaluated for its ability to detect IL-18 in rat urine samples. We examined the ability of this assay to detect IL-18 in rat urine samples spiked with known levels of the protein, and determined that dilutional linearity could be produced utilizing these samples. However, this ELISA failed to detect IL-18 using D-serine rat samples predicted to elicit a strong IL-18 response (200 mg/kg D-serine dose 24 and 48 hour post-dose) failed to detect IL-18. Other than published research indicating that IL-18 increases in a cisplatin-induced acute kidney injury model examining whole-kidney homogenates (Faubel et al., 2007), little data exists on the properties of IL-18 biomarker reactivity in other nephrotoxin models. Therefore, while the assay is adequate to detect IL-18, no protein was detected in the first evaluations of samples from the D-serine rat study. This may be due to several possible factors: 1. no IL-18 is released upon damage; 2. the level of kidney damage was too low to initiate significant IL-18 release; or 3. IL-18 may be released, but at a much later time period than tested. As the focus of this study was the prevalidation of *early* markers of subclinical damage, it was decided not to further examine IL-18 levels in the remaining urine samples.

The data obtained from this study indicate that IL-18 may not be expressed and released at significant levels in response to D-serine nephrotoxin injury. Therefore, while IL-18 has been shown to be an excellent biomarker to acute tubular necrosis, its use may not be reliable as a kidney injury biomarker due to low-level toxin exposures, and further research will be required to include or discount this protein as a potential biomarker to subclinical kidney damage in a nephrotoxin model.

2.14.2 Interleukin-18 Introduction

Interleukin 18 (IL-18) is produced as a 24 kDal inactive preprotein which is specifically cleaved by caspase-1 to produce the active 18 kDal form (Gracie et al., 2003). Interleukin 18 (interferon-gamma-inducing factor) has been characterized as a pro-inflammatory cytokine constitutively expressed in the distal tubules but which is also induced upon injury in the proximal tubule segment of the kidney. IL-18 induces the IFN-gamma production of T Cells (Tschoeke et al., 2006). IL-18 can also inhibit neutrophil apoptosis during systemic inflammation (Melnikov et al., 2002) and this increase can elicit a cellular effort to counteract neutrophil-related tissue damage and the comminant organ damage (Hirata et al., 2008) as well as mediate

116

ischemic acute tubular necrosis (Parikh et al., 2004). In the kidney, IL-18 can be over expressed in the tubular epithelial cells as a response to kidney damage due to diabetes. This activation is thought to occur by means of the induction MAPK pathway via TGF-beta (Miyauchi et al., 2009). Published data indicate that this IL-18 response is also indicative of kidney injury in some (pre-renal azotemia, urinary tract infection, chronic renal insufficiency and nephritic syndrome), but not all, kidney injury diseases (Parikh et al., 2004; Haase et al., 2008). IL-18 has been found in the urine upon acute kidney injury (Melinikov et al., 2001), and several studies are examining the use of this protein as an early response urinary biomarker to kidney damage (Endre et al., 2008).

2.14.3 Interleukin-18 Methods, Assumptions, and Procedures

Urine Collection D-serine Study 1

Male Fischer 344 rats were dosed with 5, 20, 50, 200 or 500 mg/kg D-serine in saline, whereas control animals were dosed with saline alone. Five animals were included in each dosing group with the exception of the 200 mg/kg group in which only four animals were included. Urine was collected in metabolic cages from animals prior to dosing and at 24, 48, 72 and 96 hours post-dosing. One percent sodium azide, a bacteriostatic preservative, was added to each sample, and urine was frozen at -20°C within 1 hour of end point collection.

Rat IL-18 ELISA

Enzyme-linked immunosorbent assays were performed as described in the BioSource International Rat Interleukin-18 kit protocol (BioSource International, CA). Standards, quality controls and samples were prepared in standard diluent buffer. One hundred microliters of diluted sample were added to the wells of 96-well microtiter plates coated with rat IL-18 antibody. Fifty microliters of incubation buffer were added to each well, and the plates were incubated for 2 hours at room temperature. Plates were washed 4 times with wash buffer. One hundred microliters of biotinylated anti-IL-18 conjugate were added and incubated for 1 hour at room temperature. Wells were washed four times, 100 uL of streptavidin-HRP working solution was added to the wells, and plates were incubated for 30 minutes at room temperature. Wells were again washed four times, 100 uL of stabilized TMB chromogen was added to the wells, and plates were incubated for 30 minutes at room temperature in the dark. Reactions were stopped by the addition of 100 uL of stop solution (hydrochloric acid). The absorbance (optical density [OD]) was measured at 450 nm with a microplate reader (Molecular Devices SpectraMax M2[e]).

Rat IL-18 ELISA Kit Evaluation1

For one urine sample expected to have measurable levels of IL-18 (200 mg/kg, 48 hours post-dose), an 8-point, 2-fold serial dilution was performed, beginning at 1:10 and ending at 1:1280. Reference standard material at concentrations of 0, 40 and 400 pg/mL of IL-18 were spiked into a baseline urine sample diluted 1:20, 1:100 and 1:1000.

Rat IL-18 ELISA Kit Evaluation 2

For two urine samples expected to have measurable levels of IL-18 (24 hours post-dose, 200 and 500 mg/kg), a 3-point, 2-fold serial dilution was performed, beginning at 1:4 and ending at 1:16.

2.14.4 Interleukin-18 Results and Discussions

Rat IL-18 ELISA Kit Evaluation

The BioSource International IL-18 ELISA kit is validated for the quantitative determination of IL-18 in rat serum, EDTA plasma, buffered solution and cell culture medium, only. Therefore, validation of this kit in a rat urine matrix was necessary prior to its use. The kit was evaluated by three independent analyses in order to assess the kit's capability of detecting IL-18 in rat urine. First, the recovery efficiency of IL-18 from the urine matrix was assessed through a spike and recovery experiment. Second, the kit's capability of detecting IL-18 from in-house rat urine samples expected to have measurable levels of the protein was assessed. Third, the linearity of the results obtained from multiple dilutions of a urine sample was assessed.

Rat IL-18 ELISA Kit Evaluation 1

The kit insert did not recommend a starting dilution of samples. Therefore, the first evaluation was designed to assay IL-18 using rat urine samples at multiple dilutions to establish the appropriate sample dilution. Additionally, a spike and recovery experiment was performed to assess the recovery efficiency of IL-18 from the urine matrix. Various dilutions of a baseline urine sample were prepared in standard diluent buffer. The urine dilutions covered the range of dilutions anticipated to be used in regular sample testing. The standard material was then spiked into the urine dilutions and recovery was assessed.

No IL-18 was detected in a urine sample (200 mg/kg D-serine 48 hours post-dose) which was expected to contain measurable levels of the protein. For all eight dilutions of the urine sample, the ODs fell below the range of the standard curve (data not shown). During the spike and recovery experiment, the ODs of each sample not spiked with IL-18 (0 pg/mL) fell below the standard range (Table 24). The recovery of IL-18 from spiked urine diluted 1:20 was approximately 78%. The recovery of the protein decreased to approximately 61% and 57% for urine diluted 1:100 and 1:1000, respectively. Despite decreasing recovery, the dilutional linearity between the three dilutions of the spiked samples was adequate, with intra-assay CVs of 26% and 9% for the 40 pg/mL and 400 pg/mL samples, respectively.

Table 24:   IL-18 spike and recovery of 0, 40 and 400 pg/mL IL-18

IL-18 (reference standard material) aliquoted into 1:20, 1:100 and 1:1000 dilutions of baseline urine sample

Spiked Concentration (pg/mL)	Dilution Factor	Replicate OD Values	Replicate Result (pg/mL)	Mean Result (pg/mL)	Std. Dev.	% CV	Percent Recovery
0	20	0.077	Below range	NA			
		0.085	Below range				
40		0.195	29.870	31.100	1.739	5.6	77.75%
		0.203	32.329				
400		0.963	305.560	313.893	11.785	3.8	78.47%
		0.998	322.226				
0	100	0.075	Below range	NA			
		0.070	Below range				
40		0.173	22.906	21.737	1.653	7.6	54.34%
		0.165	20.568				
400		0.848	254.307	270.991	23.595	8.7	67.75%
		0.924	287.675				
0	1000	0.070	Below range	NA			
		0.075	Below range				
40		0.142	12.933	19.204	8.870	46.2	48.01%
		0.181	25.476				
400		0.744	212.321	265.676	75.455	28.4	66.42%
		0.991	319.030				

Since no IL-18 was detected in a sample expected to contain measurable levels of the protein, the dilution experiment was repeated with two additional urine samples expected to yield measurable protein concentrations. During the spike and recovery experiment, dilutional linearity was observed between samples diluted 1:20, 1:100 and 1:1000. However, recovery of the protein from the urine matrix decreased with increasing dilution. Therefore, the dilution experiment was repeated at dilutions of 1:4, 1:8, and 1:16.

Rat IL-18 ELISA Kit Evaluation 2

Two urine samples expected to contain measureable levels of IL-18 (24 hours post-dose, 200 and 500 mg/kg dose groups) were analyzed at 1:4, 1:8 and 1:16 dilutions. At all three dilutions, the ODs of the first sample fell below the standard range of the assay. Five out of the six ODs recorded for the second sample fell within the standard range. Unlike previous analyses, no dilutional linearity was observed between the three dilutions of the urine sample (200 mg/kg – 24 hour post-dose) (Table 25). While the recovery of IL-18 spiked into various

dilutions of urine was acceptable, IL-18 was not detected in the urine samples obtained from D-serine dosed animals. Therefore, the urinary IL-18 levels do not seem to be modulated within the first 24 hours of exposure to D-sering. However, further testing at later post-dose time points may indicate that urinary IL-18 increases at a time period later than 24 hours. We were unable to complete this examination due to time and funding restraints.

Table 25: IL-18 analysis of rat urine at dilution factors of 4, 8, and 16

Replicate OD Values	Replicate Result (ng/mL)	Mean Result (ng/mL)	Std. Dev.	% CV	Dilution Factor	Adjusted Result (ng/mL)
0.126	29.043	28.259	1.109	3.9	4	113.036
0.123	27.475					
0.124	27.834	30.433	3.676	12.1	8	243.467
0.131	33.032					
0.065	Below range	35.615	NA	NA	16	569.842
0.135	35.615					

2.15 Interleukin 6 (IL-6)

2.15.1 IL-6 Summary

Several publications examining urine and serum expression of interleukin 6 (IL-6) levels confirmed that IL-6 levels are indicative of kidney damage and/or inflammation. Therefore, IL-6 was included as a potential biomarker to be examined in a D-serine rat study modeling subclinical kidney damage. In this animal study, Male Fischer 344 rats were dosed with 0, 5, 20, 50, 200, or 500 mg/kg D-serine in saline. Urine samples were kept cold during collection periods of 24, 48, 72 and 96 hours post-dose, frozen within 1 hour at each time point. Histopathological examination of kidney tissue verified the levels of renal damage at each dose. In order to quantitate IL-6 in urine, a commercially available IL-6 (Rat) EIA kit from ALPCO Diagnostics was evaluated for its ability to detect IL-6 in A rat urine test matrix. As the kit is validated for use only in rat serum, buffered solution, or cell culture medium, we examined the ability of this assay to detect IL-6 in rat urine samples spiked with known levels of the protein. It was demonstrated that dilutional linearity could not be produced with this kit using a rat urine test matrix. In addition, samples of the highest IL-6 level QC sample fell below acceptable range established by the manufacturer. As the kit failed validation, we were unable to examine the time/dose response expression of IL-6 in the developed D-serine nephrotoxin samples. While some urine matrix interference may have affected test results, it was felt that the EIA did not perform as well as claimed by the manufacturer. Therefore, our examination of IL-6 as a possible biomarker indicative of subclinical kidney damage was neither proved nor disproved, and must wait the development of a rat-specific ELISA capable of sensitive detection in a urine matrix.

2.15.2 IL-6 Introduction

Interleukin 6 (IL-6) is a 23 kDal peptide cytokine produced in a wide range of cell types (Kishimoto et al., 1995), and has been shown to play a role in both the proinflammatory and anti-inflammatory processes. In addition, this cytokine has been shown to induce production of acute phase proteins in response to damage as well as act as a growth inducer to stimulate the production of renal mesangial cells (Castell et al., 1988).

As renal failure stimulates the inflammatory response in the kidney, studies in renal failure patients noted an increase in IL-6 levels in the serum (Herbelin et al., 1991; Kaysen et al., 2003). Additionally, IL-6 has been shown to increase in plasma in dialysis patients, thought to be caused by an inflammatory response stimulated in the kidney due to interactions with hemodialysis membranes (Memoli et al., 1992). Recent data examining levels of several cytokines in the serum in a bead-based multiplex cytokine chip demonstrated that serum IL-6 can identify AKI very early in patients undergoing cardiopulmonary bypass surgery. IL-6 is also released into the blood upon prolonged usage of mechanical ventilation (Liu et al., 2009). The use of IL-6 as a urinary marker of kidney injury was examined in a Lupus nephritis model and levels were shown to be substantially higher in patients exhibiting this secondary kidney effect (Li et al., 2006; Iwano et al., 1993).

2.15.3 IL-6 Methods, Assumptions, and Procedures

Urine Collection D-serine Study 1

Male Fischer 344 rats were dosed with 5, 20, 50, 200 or 500 mg/kg D-serine in saline, whereas control animals were dosed with saline alone. Five animals were included in each dosing group with the exception of the 200 mg/kg group in which only four animals were included. Urine was collected in metabolic cages from animals prior to dosing and at 24, 48, 72 and 96 hours post-dosing. One percent sodium azide, a bacteriostatic preservative, was added to each sample, and urine was frozen at -20°C within 1 hour of end point collection.

IL-6 (Rat) EIA

Enzyme-linked immunosorbent assays were performed as described in the ALPCO Diagnostics IL-6 (Rat) EIA kit protocol (ALPCO Diagnostics, NH). Standards, quality controls and samples were prepared in standard diluent buffer. One hundred microliters of diluted sample were added to the wells of 96-well microtiter plates coated with rat IL-6 antibody, and the plates were incubated for 2 hours at 37 °C. Plates were washed 4 times with wash buffer. One hundred microliters of biotinylated anti-IL-6 conjugate were added and incubated for 1 hour at room temperature. Wells were washed four times, 100 uL of streptavidin-HRP working solution was added to the wells, and plates were incubated for 30 minutes at room temperature. Wells were washed four times, 100 uL of stabilized TMB chromogen was added to the wells, and plates were incubated for 30 minutes at room temperature in the dark. Reactions were stopped by the addition of 100 uL of stop solution (phosphoric acid). The absorbance (optical density [OD]) was measured at 450 nm with a microplate reader (Molecular Devices SpectraMax M2^e).

IL-6 (Rat) EIA Kit Evaluation 1

For four urine samples expected to have measurable levels of IL-6 (200 mg/kg-48 and 96 hours post-dose, 500 mg/kg-24 and 48 hour post-dose), 2-point 10-fold serial dilutions were performed, beginning at 1:4 and ending at 1:40. Reference standard material at concentrations of 0, 100 and 1000 pg/mL of IL-6 was spiked into a baseline urine sample diluted 1:4 and 1:40.

IL-6 (Rat) EIA Kit Evaluation 2

For seven urine samples expected to have measurable levels of IL-6 (500 mg/kg-24and 96 hour, 500 mg/kg-24 hour, 200 mg/kg-24 and 96 hour, 150 mg/kg-24 hour, and 150 mg/kg-24 hour), 2-point, 2-fold serial dilutions were performed, beginning at 1:8 and ending at 1:16.

2.15.4 IL-6 Results and Discussions

IL-6 (Rat) EIA Kit Evaluation

The ALPCO Diagnostics IL-6 EIA kit is validated for the quantitative determination of IL-6 in rat serum, buffered solution and cell culture medium, but not in urine. Therefore, the kit was evaluated by three independent analyses in order to assess the kit's capability of detecting IL-6 in rat urine. First, the recovery efficiency of IL-6 from the urine matrix was assessed

through a spike and recovery experiment. Second, the kit's capability of detecting IL-6 from in-house rat urine samples expected to have measurable levels of the protein was assessed. Third, the linearity of the results obtained from multiple dilutions of a urine sample was assessed.

IL-6 (Rat) EIA Kit Evaluation 1

The kit insert did not recommend a starting dilution of samples. Therefore, the first evaluation was designed to assay urine samples expected to have relatively high levels of IL-6 at multiple dilutions to establish the appropriate sample dilution. Additionally, a spike and recovery experiment was performed to assess the recovery efficiency of IL-6 from the urine test matrix. Various dilutions of a baseline urine sample were prepared in standard diluent buffer and covered the range of dilutions anticipated to be used in regular sample testing. The protein standard was used to spiked urine and its recovery was assessed. It should be noted that the QC sample with the highest concentration of test protein fell below the acceptable range established by the manufacturer.

No IL-6 was detected in the urine samples expected to contain measurable levels of the protein. For both dilutions of the samples, the ODs fell below the range of the standard curve (data not shown). Rather than see a decrease in ODs as the dilution factor increased, no significant changes in ODs were observed. Consequently, dilutional linearity was not observed. During the spike and recovery experiment, approximately 40% of the spiked protein was recovered from urine diluted 1:4 while approximately 77% of the spiked protein was recovered from urine diluted 1:40 (Table 26).   Because the QC sample included in the kit fell below the acceptable range established by the manufacturer and no IL-6 was detected in samples expected to contain measurable levels of the protein, the dilution experiment was repeated with additional urine samples expected to yield measurable protein concentrations.

Table 26: IL-6 spike and recovery of 0, 100 and 1000 pg/mL
IL-6 (reference standard material) was aliquoted into 1:4 and 1:40 dilutions of baseline urine sample

Spiked Concentration (pg/mL)	Dilution Factor	Replicate OD Values	Replicate Result (pg/mL)	Mean Result (pg/mL)	Std. Dev.	% CV	Percent Recovery
0	4	0.140	Below range	NA	NA	NA	0
		0.150	Below range				
100		0.235	43.584	40.193	4.796	11.9	40.2
		0.222	36.802				
1000		0.856	402.982	403.390	0.577	0.1	40.3
		0.858	403.798				
0	40	0.149	Below range	NA	NA	NA	0
		0.146	Below range				
100		0.293	75.429	78.200	3.919	5.0	78.2
		0.303	80.972				
1000		1.374	758.627	757.157	2.079	0.3	75.7
		1.370	755.687				

IL-6 (Rat) EIA Kit Evaluation 2

      Seven urine samples expected to contain measureable levels of IL-6 were analyzed at 1:8 and 1:16 dilutions, and at both dilutions, the ODs of all samples fell below the standard range of the assay (Figure 67). As previously observed, the QC sample again fell below the acceptable range established by the manufacturer. IL-6 was not detected in the urine study samples. Therefore, the IL-6 (Rat) EIA kit from ALPCO Diagnostics was not included in the study as it failed multiple validation analyses. While IL-6 may indeed be an adequate biomarker to subclinical renal damage, such a determination must await development of a quantitative IL-6 assay suitable for use in a urine test matrix.
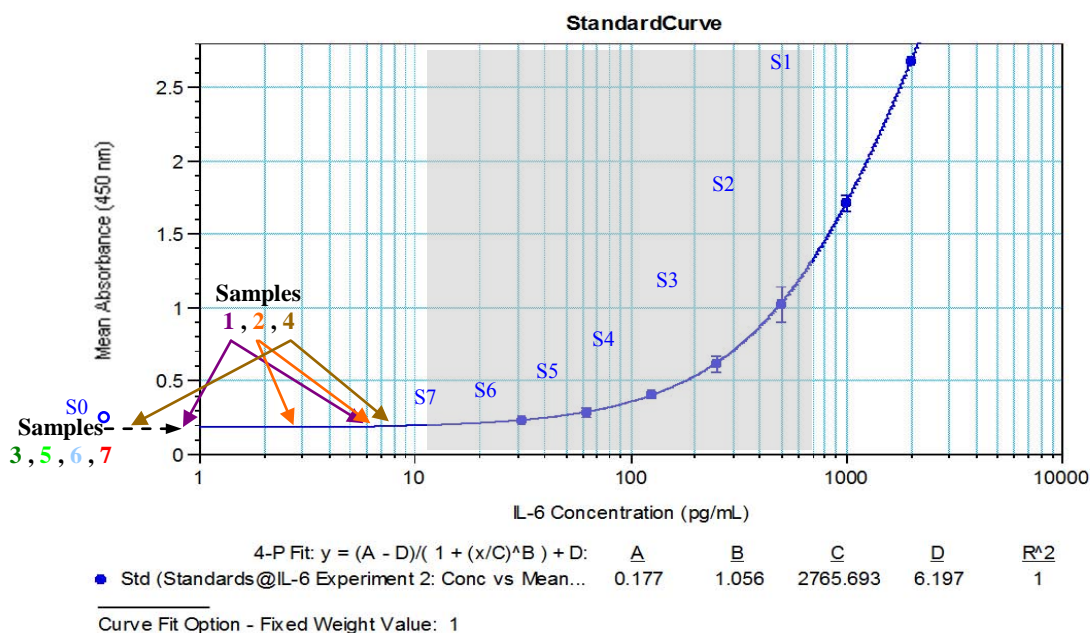
Figure 67: IL-6 standard curve depicting assay range and concentrations observed in seven urine samples

2.16 proAtrial Natriuretic Peptide (proANP)

2.16.1 ANP Summary

Many published reports have indicated that atrial natriuretic peptides (proANP) are released into the urine and blood upon renal injury,which is seen as well as in several cardiovascular diseases. As the use of proANP as a potential biomarker to renal injury is strongly supported by data, it was chosen for further examination as a potential biomarker in a nephrotoxin (D-serine and puromycin) rat study modeling subclinical kidney damage. To initiate low level kidney damage at the kidney tubule or glomerulus, Male Fischer 344 rats were dosed with 0, 5, 20, 50, 200, or 500 mg/kg D-serine in saline or 0, 5, 25, 75, or 150 mg/kg puromycin in saline. Urine samples were kept cold during collection periods of 24, 48, 72 and 96 hours post-dose, and frozen within 1 hour of collection at each time point. Histopathological examination of dosed kidney tissue verified the levels of damage. To quantitate proANP in the samples, a commercially available assay (Biomedica proANP (1-98) ELISA) was evaluated for its ability to detect fibronectin in urine samples using test samples spiked with known quantities of the protein. The dilutional linearity between the two dilutions of the spiked samples was adequate, with an intra-assay CV of 17%, with recovery of the protein ranging from 64 to 76% in urine depending upon dilution. However, the ODs of all samples fell below the standard range of the assay. Therefore, while the assay is adequate to detect proANP, no protein was detected in the first evaluations of samples from the D-serine rat study. This may be due to several possible factors: 1. no proANP is released upon damage; 2. the level of kidney damage was too low to initiate significant proANP release; or 3. proANP may be released, but at a much later time period than tested. As the focus of this study was the prevalidation of *early* markers of subclinical damage, it was decided not to examine proANP levels in the remaining urine samples. While proANP may be released in response to D-serine or puromycin exposures, the initial assay data indicated that release of this natriuretic peptide would occur at time periods greater than 48 hours in the D-serine model or greater than 168 hours in the puromycin model or require nephrotoxin concentrations that stimulate kidney damage greater than the subclinical levels examined in this study.

2.16.2 ANP Introduction

Atrial natriuretic peptide (proANP) is produced as a 126 amino acid prohormone primarily in atrial myocytes, but it has also been shown to be synthesized in the kidney (Vesely 1992). The prohormone form consists of four hormones with different properties: proANP (aa 1-30) long acting natriuretic peptide; proANP (aa 31-67) a vessel dilator; proANP (aa 79-98) a kaliuretic peptide; and ANP (aa 99-126). All four proANP peptide hormones have been shown to be synthesized in the kidney.

Atrial natriuretic peptide (proANP) and brain natriuretic peptide (BNP) are both cardiac natriuretic peptides levels whose levels been shown to increase in the plasma during renal dysfunction, hypertension, as well as heart disease (Cataliotti et al., 2001; excellent review in Vesely et al., 2003; Horl et al., 2005). Both peptides have been shown to be linked to left ventricular mass of the heart and are considered a viable biomarker to diagnose cardiovascular injury (Tripepi et al., 2009; Mallamaci et al., 2001). Both also increase in patients on peritoneal dialysis versus hemodialysis, possibly due to renal injury due to dialysis membranes (Suresh et al., 2005; Racek et al., 2006).   Elevated plasma ANP levels have been shown to increase during

hemodialysis in patients with chronic renal failure (Takagi et al., 1993; Haug et al., 1994). The neural endopeptidase system has been shown to exist in renal tubular epithelial cells, so it is thought that renal injury leads to a loss of this natriuretic peptide degradative pathway resulting in elevated natriuretic peptides in serum as well as in urine (Metry et al., 2001; Franz et al., May 2001; Franz et al., Jul 2001).

2.16.3 proANP Methods, Assumptions, and Procedures

Urine Collection D-serine Study 1

Male Fischer 344 rats were dosed with 0, 5, 20, 50, 200 or 500 mg/kg D-serine in saline. Five animals were included in each dosing group with the exception of the 200 mg/kg group in which only four animals were included. Urine was collected in metabolic cages from animals prior to dosing and at 24, 48, 72 and 96 hours post-dose. One percent sodium azide, a bacteriostatic preservative, was added to each sample, and urine was frozen at -20°C within 1 hour of end point collection.

proANP (1-98) EIA Protocol

Enzyme-linked immunosorbent assays were performed as described in the Biomedica proANP (1-98) ELISA kit protocol (Biomedica Gruppe, Vienna, Austria). Standards and quality controls were prepared in distilled water. Samples were diluted in assay buffer and 10 uL of diluted sample were added to the wells of 96-well microtiter plates coated with polyclonal sheep anti-proANP antibodies. Two hundred microliters of HRP-labeled polyclonal anti-proANP antibody conjugate were added, and the plates were incubated for 3 hours at room temperature (18-26 °C) in the dark. Plates were washed 5 times with wash buffer, 200 uL of TMB substrate were added to the wells, and plates were incubated for 30 minutes at room temperature in the dark. Reactions were stopped by the addition of 50 uL of stop solution (sulfuric acid). The absorbance (optical density [OD]) was measured at 450-620 nm with a microplate reader (Molecular Devices SpectraMax M2[e]).

proANP (1-98) EIA Kit Evaluation1

For two urine sample expected to have measurable levels of proANP (50 mg/kg-48 hour and 500 mg/kg-24 hour), 4-point, and 2-fold serial dilutions were performed, beginning at 1:1 and ending at 1:8. Reference standard material at concentrations of 0 and 2 nmol/L of proANP were spiked into a baseline urine sample diluted 1:1 and 1:10.

proANP (1-98) EIA Kit Evaluation 2

Four urine samples expected to have measurable levels of proANP were analyzed undiluted and at a 1:5. Samples included two animal samples from 200 mg/kg collected 96 hour post-dose (D-serine) as well as one sample from 150 mg/kg -72 hour post-dose and one sample from 150 mg/kg -168 hour post-dose (puromycin).

proANP (1-98) EIA Kit Evaluation 3

Four urine samples expected to have measurable levels of proANP (150 mg/kg dose, 24, 48, 96, and 120 hour collection) were analyzed undiluted and at a 1:2 were tested.

2.16.4 proANP Results and Discussions

The Biomedica proANP (1-98) ELISA kit is validated for the quantitative determination of human proANP in EDTA plasma, heparin plasma, urine and cell culture medium. The kit cross-reacts with rat, but has not been validated for use with rat urine. Therefore, the kit was evaluated by three independent analyses in order to assess the assay capability of detecting proANP in rat urine. First, the recovery efficiency of proANP from the urine matrix was assessed through a spike and recovery experiment. Second, the kit's capability of detecting proANP from in-house rat urine samples expected to have measurable levels of the protein was assessed. Third, the linearity of the results obtained from multiple dilutions of a urine sample was assessed.

proANP (1-98) EIA Kit Evaluation 1

The kit insert recommended testing urine samples neat and diluting samples in assay buffer when necessary. The first evaluation was designed to assay two urine samples expected to have relatively high levels of proANP at multiple dilutions (1:1, 1:2, 1:4 and 1:8) to establish the appropriate sample dilution. Additionally, a spike and recovery experiment was performed to assess the recovery efficiency of proANP from the urine matrix. A baseline urine sample was diluted 1:1, 1:2, 1:4 and 1:8 in assay buffer. The standard material was then spiked into the urine dilutions and recovery was assessed. No proANP was detected in the urine sample expected to contain measurable levels of the protein. For all four dilutions of the two urine samples, the ODs fell below the range of the standard curve (Figure 68). Rather than seeing a decrease in ODs as the dilution factor increased, no significant changes in ODs was observed. Consequently, dilutional linearity was not observed.

127
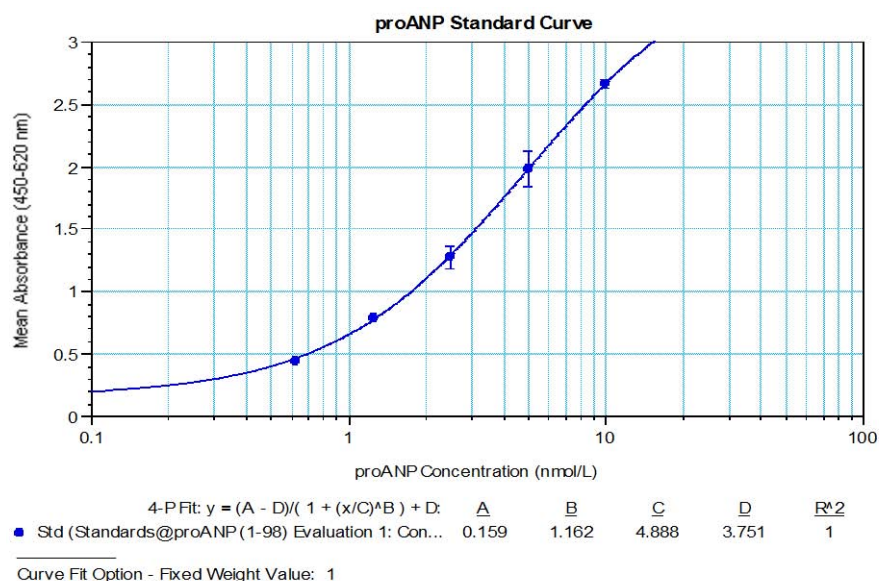
Figure 68: proANP Standard Curve

During the spike and recovery experiment, the ODs of each sample not spiked with proANP (0 pg/mL) fell below the standard range (Table 27). The recovery of proANP from spiked urine diluted 1:1.125 was approximately 64%. The recovery of the protein increased to approximately 76% for urine diluted 1:10. The dilutional linearity between the two dilutions of the spiked samples was adequate, with an intra-assay CVs of 17%. Since no proANP was detected in samples expected to contain measurable levels of the protein, the dilution experiment was repeated with four additional urine samples expected to yield measurable protein concentrations.

Table 27: proANP spike and recovery of 0 and 2 nmol/L

proANP (reference standard material) was aliquoted into 1:1 and 1:10 dilutions of baseline urine sample. Urine sample used was 500 mg/kg D-serine pre-dose

Spiked Concentration (nmol/L)	Dilution Factor	Replicate OD Values	Replicate Result (nmol/L)	Mean Result (nmol/L)	Std. Dev.	%CV	Percent Recovery
0	1	0.177	Below range	NA			
		0.181	Below range				
2	1.125	0.773	1.257	1.278	0.029	2.3	63.9%
		0.792	1.298				
0	10	0.257	Below range	NA			
		0.157	Below range				
2		0.903	1.540	1.516	0.038	2.3	75.8%
		0.881	1.491				

proANP (1-98) EIA Kit Evaluation 2

Four urine samples expected to contain measureable levels of proANP were analyzed undiluted and at 1:5 dilution. The ODs of all four samples tested fell below the standard range of the assay (data not shown). The experiment was repeated at a lower dilution with an additional four samples expected to contain measurable concentrations of the protein.

proANP (1-98) EIA Kit Evaluation 3

Four urine samples expected to contain measureable levels of proANP were analyzed undiluted and at 1:2. As previously observed, the ODs of all samples fell below the standard range of the assay (data not shown).

While the ELISA used was adequate for quantitiation of proANP, preliminary testing indicated that no proANP could be detected in the urine from either D-serine or puromycin dosed animals. Therefore, as proANP was not detected in any of the urine study samples, further studies on proANP (1-98) response were not conducted.

2.17 Fibronectin

2.17.1 Fibronectin Summary

Fibronectin levels have been shown to increase in response to cellular injury as seen in several kidney disease models. This evidence provided support that fibronectin protein expression may increase upon exposure to nephrotoxins with release into the urine. While there are few studies examining this protein in urine and serum in nephrotoxin models (Nangaku et al., 1999 being the exception), fibronectin was included as a possible biomarker to be examined in the D-serine rat study modeling subclinical kidney damage. To model early kidney damage, Male Fischer 344 rats were dosed with 0, 5, 20, 50, 200, or 500 mg/kg D-serine in saline. Urine samples were kept cold during collection periods of 24, 48, 72 and 96 hours post-dose, and frozen within 1 hour of collection. A commercially available assay, the Assaypro AssayMax Rat Fibronectin ELISA, was evaluated for its ability to detect fibronectin in urine samples using test samples spiked with known quantities of the protein. Several evaluations of this kit determined that dilutional linearity could not be reproduced in a rat urine test matrix. It was also shown that urine provided significant binding interference that affected test results. Since the dilutional linearity was unsatisfactory and thus the ability of the kit to detect fibronectin in urine samples was questionable, fibronectin ELISA testing was not pursued further in this study. Since our data is inconclusive due to assay failure in a urine test matrix, data on the utility of fibronectin as a urinary biomarker to subclinical kidney damage induced by nephrotoxicants must await the development of a new rat-specific fibronectin assay with far lower levels of urine interference.

2.17.2 Fibronectin Introduction

Structurally, fibronectin consists of three homologous repeating modules, designated Type I, II, and III, which can form up to two disulfide bridges at their C-terminus, giving the protein the capability of forming dimers (Potts et al., 1994; Potts et al., 1996). Fibronectin has been shown to be produced as two different isoforms: a plasma protein (produced by hepatocytes) and an extracelluar matrix protein (produced by fibroblasts, chondrocytes, endothelial and epithelial cells). This protein has been identified as playing a role in tissue repair, blood clotting, as well as cell adhesion activities.

Fibronectin has been examined as a possible urinary biomarker in bladder cancer (Malmström et al., 1993) and as a plasma biomarker for preeclampsia (Yasumizu et al., 1996) although a thorough interrogation as to its utility as an indicator to kidney injury and AKI has only been recently explored. Immunostaining data using tubular damage induced by the nephrotoxin puromycin in glaxo (PVG) rats demonstrated the accumulation of fibronectin in the extracellular matrix at regions near areas of damage (Nangaku et al., 1999). The accumulation of fibronectin in the glomerulus was shown in several kidney injury models (obstructed kidney, toxin damage, glomerulosclerosis) and has been adapted as a diagnostic indicator (Bruneval et al., 1985; Zhang et al., 2008). This induction of fibronectin and other extracellular proteins in glomerular mesangial cells has been shown to be initiated by mitogen-activated protein kinase (MAPK) activated by glomerular cell 'stretching' due to hypertension induced by injury or disease (Ishida et al., 1999). This 'stretching' was also shown to increase fibronectin mRNA with concomitant protein production and accumulation at the glomerulus. Upregulation of fibronectin

mRNA levels were also seen in interstitial fibrosis in the obstructed kidney (Ma et al., 2009), perhaps by an equivalent mechanism.

2.17.3 Fibronectin Methods, Assumptions, and Procedures

Urine Collection D-serine Study 1

Male Fischer 344 rats were dosed with 5, 20, 50, 200 or 500 mg/kg D-serine in saline, whereas control animals were dosed with saline alone. Five animals were included in each dosing group with the exception of the 200 mg/kg group in which only four animals were included. Urine was collected in metabolic cages from animals prior to dosing and at 24, 48, 72 and 96 hours post-dosing. One percent sodium azide, a bacteriostatic preservative, was added to each sample, and urine was frozen at -20°C within 1 hour of end point collection.

Rat Fibronectin ELISA

Enzyme-linked immunosorbent assays were performed as described in the Assaypro AssayMax Rat Fibronectin ELISA kit protocol (Assaypro LLC, MO). Standards and samples were prepared in EIA diluent. Fifty microliters of diluted sample were added to the wells of 96-well microtiter plates coated with polyclonal antibody against rat fibronectin, and the plates were incubated for 2 hours at room temperature (20-30 °C). Plates were washed 5 times with wash buffer, and 50 uL of biotinylated fibronectin polyclonal antibody were added and incubated for 1 hour at room temperature. Wells were washed five times, 50 uL of streptavidin-peroxidase conjugate were added to the wells, and plates were incubated for 30 minutes at room temperature. Wells were again washed five times, 50 uL of stabilized peroxidase chromogen TMB substrate added to the wells, and plates incubated for 10 minutes at room temperature. Reactions were stopped by the addition of 50 uL of 0.5 N hydroxychloric acid. The absorbance (optical density [OD]) was measured at 450 nm with a microplate reader (Molecular Devices SpectraMax M2[e]).

Rat Fibronectin ELISA Kit Evaluation 1

For five urine samples expected to have measurable levels of fibronectin (200 mg/kg-24 and 48 hour, 200 mg/kg-48 and 96 hour, 500 mg/kg-24 and 48 hour), 2-point, 10-fold serial dilutions were performed, beginning at 1:4 and ending at 1:40. Reference standard material at concentrations of 0, 0.1 and 1 ug/mL of fibronectin was spiked into a baseline urine sample diluted 1:4 and 1:40.

Rat Fibronectin ELISA Kit Evaluation 2

For two urine samples expected to have measurable levels of fibronectin (24 hours post-dose collection, 200 and 500 mg/kg dose) 4-point, 2-fold serial dilutions were performed, beginning at 1:8 and ending at 1:64.

2.17.4 Fibronectin Results and Discussions

Rat Fibronectin ELISA Kit Evaluation

The Assaypro AssayMax rat fibronectin ELISA kit is validated for the quantitative determination of fibronectin in rat serum, plasma and cell culture medium, but not in urine. Therefore, the kit was evaluated by two independent analyses in order to assess the kit's capability of detecting fibronectin in rat urine. First, the recovery efficiency of fibronectin from the urine matrix was assessed through a spike and recovery experiment. Second, the kit capability of detecting fibronectin from Study 1 rat urine samples expected to have measurable levels of the protein was assessed. Third, the linearity of the results obtained from multiple dilutions of a urine sample was assessed.

Rat Fibronectin ELISA Kit Evaluation 1

The first evaluation was designed to assay urine samples expected to have relatively high levels of fibronectin at two dilutions (1:4 and 1:40) to establish the appropriate sample dilution. Additionally, a spike and recovery experiment was performed to assess the recovery efficiency of fibronectin from the urine matrix. A baseline urine sample was diluted 1:4 and 1:40 in EIA diluent. The standard material was then spiked into the urine dilutions and recovery was assessed. The OD range of the reference standard was very low (Figure 69), ranging from an OD of only 0.300 for the highest standard (2 ug/mL) to an OD of 0.070 for the lowest standard (0.032 ug/mL). A QC report for the reference standard was not provided with the kit. A standard curve with such a narrow range is not satisfactory, given that the CV of replicate wells functions as an acceptance criterion, and the lower the OD, the easier it is to "fail" acceptable data.
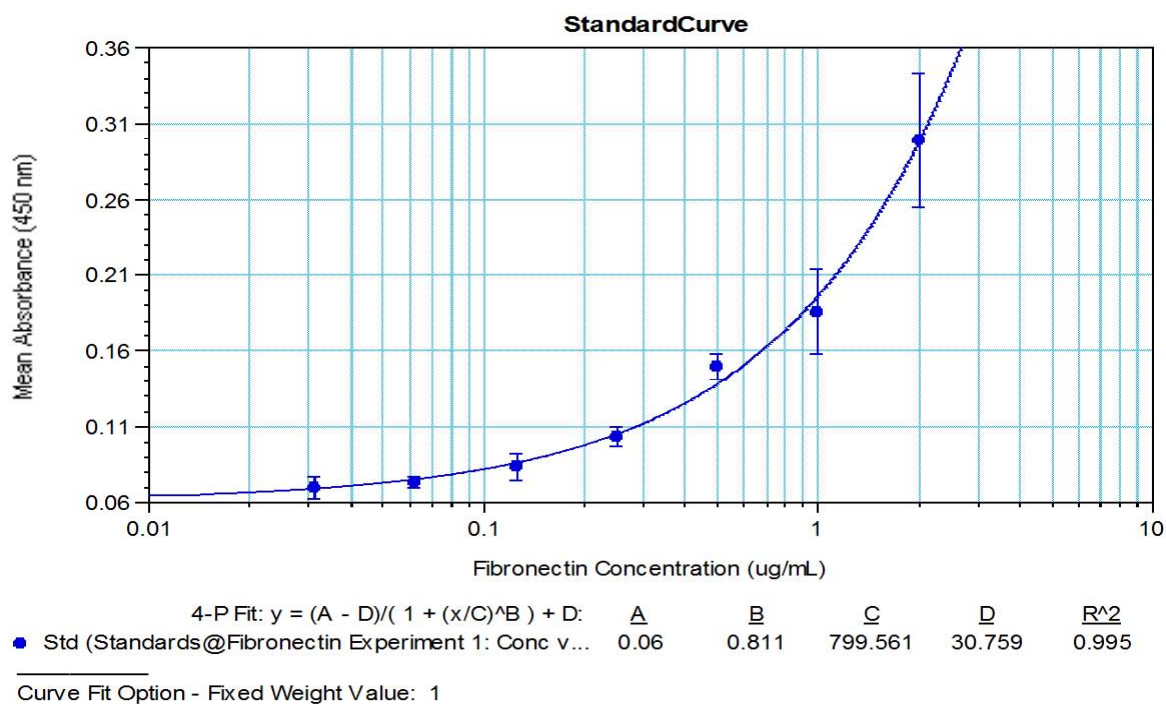
Figure 69: **Fibronectin standard curve**

The recovery of fibronectin from spiked urine diluted 1:40 was approximately 70% (Table 28). The recovery of the protein decreased to approximately 46% for urine diluted 1:4. This suggests interference of the urine matrix with fibronectin binding at the 1:4 dilution.

Table 28: Fibronectin spike and recovery of 0, 0.1 and 1 ug/mL

Fibronectin (reference standard material) was aliquoted into 1:4 and 1:40 dilutions of baseline urine sample (200 mg/kg pre-dose)

Spiked Concentration (µg/mL)	Replicate OD Values	Replicate Result (µg/mL)	Mean Result (µg/mL)	Dilution Factor	Percent Recovery
0	0.064	Below Range	NA	4	NA
	0.062	Below Range			
0.1	0.074	0.059	0.059	4	59
	0.069	Below Range			
1	0.108	0.277	0.330	4	33
	0.123	0.383			
0	*0.066	Below Range	NA	40	NA
	*0.063	Below Range			
0.1	0.073	0.055	0.066	40	66
	0.078	0.078			
1	0.167	0.747	0.750	40	75
	0.168	0.753			

For four out of the five urine samples, the ODs fell within the range of the standard curve (Table 29). However, dilutional linearity was not observed between the 1:4 and 1:40 dilutions. This is to be expected since the results of the spike and recovery experiment suggested matrix interference at the 1:4 dilution. A second evaluation experiment was performed to reassess the OD range of the reference standard and to examine more closely the dilutions between 1:4 and 1:40 as well as one dilution point beyond 1:40.

Table 29: Fibronectin analysis of rat urine at dilution factors of 4 and 40

Sample	Dilution Factor	Mean OD	Mean Result (µg/mL)	Duplicate % CV	Adjusted Result
1 200 mg/kg-24hr post-dose	4	0.100	0.220	72.2	0.880
	40	0.080	0.092	54.3	3.690
2 200 mg/kg-48hr post-dose	4	0.073	0.050	0.7	0.201
	40	0.065	Below range	NA	NA
3 200 mg/kg-96hr post-dose	4	0.069	Below range	NA	NA
	40	0.066	Below range	NA	NA
4 500 mg/kg-24hr post-dose	4	0.098	0.201	13.2	0.803
	40	0.083	0.106	23.0	4.246
5 500 mg/kg-48hr post-dose	4	0.085	0.122	1.1	0.487
	40	0.073	0.050	26.8	2.018

134

Rat Fibronectin ELISA Kit Evaluation 2

Two urine samples (samples 1 and 4 from the previous evaluation) were analyzed at 1:4, 1:8, 1:16, 1:32 and 1:64 dilutions (Table 30). While the ODs of both samples at all four dilutions fell within the standard range of the assay, no dilutional linearity was observed between the obtained results. Since the ODs at each dilution remained nearly constant instead of decreasing as a function of dilution, it is likely that the ODs are a result of background signal rather than fibronectin binding. It should also be pointed out that the OD range of the reference standard was again very low, ranging from an OD of only 0.262 for the highest standard to an OD of 0.075 for the lowest standard.

Since the narrow range of the reference standard is not satisfactory and given that the ability of the kit to detect fibronectin in urine samples is inconclusive, the Assaypro AssayMax rat fibronectin ELISA data was not pursued further in this study. Therefore, examination of fibronectin as a biomarker to subclinical renal damage must await the development of an assay insensitive to interference from the urine test matrix.

Table 30: Fibronectin analysis of rat urine at dilution factors of 8, 16, 32 and 64

Sample	Dilution Factor	OD Value	Result (µg/mL)	Adjusted Result (µg/mL)
1 200 mg/kg 24 hour post-dose	8	0.104	0.225	2.042
	16	0.082	0.062	0.994
	32	0.093	0.158	5.048
	64	0.103	0.250	15.974
2 500 mg/kg 24 hour post-dose	8	0.122	0.435	3.478
	16	0.123	0.443	7.086
	32	0.130	0.518	16.583
	64	0.081	0.058	3.682

3.2 Group Specific Component (GSC)

3.2.1 GSC Summary

Urine proteomics analysis by RHPB Proteomics has identified the protein Group Specific Component (GSC), also called the Vitamin D binding protein, as a potential biomarker to early subclinical kidney degradation using a D-serine nephrotoxin renal injury model. Increased expression of a protein containing significant homology to human and rat GSC was identified in the urine of rats dosed with 500 mg/kg D-serine (Section 3.1.1.1, Shiyanov et al., 2009). Based on proteomics data identifying this as a potential biomarker, assays were developed to GSC to obtain the expression profile of this protein in both serum and urine from rats injected with subtoxic levels of D-serine. To model subclinical renal toxin injury, low levels of D-serine were used to induce kidney damage at the proximal straight tubules. Male Fischer 344 rats were dosed intraperitoneally with control (0), 200, or 500 mg/kg D-serine in 0.9% saline and urine collected pre-dose and at timed increments post-dose. Urine was collected from animals and frozen within 1 hour of end point collection. Renal damage was verified by histopathological examination of kidney tissue collected at sacrifice. To develop a rat-specific GSC assay, two peptides based on rat GSC protein sequence were synthesized and used as antigens for polyclonal antibody development in the rabbit. These two polyclonals, designated GSC 1239 and GSC 1242 were raised against residues 49-61 and 436-451, respectively, of rat GSC (Access No. Q68FY4). Western blots using GSC 1242 tested against urine from a 500 mg/kg, 24 hour post-dose animal from the D-serine study demonstrated a strong signal to a 52 kDal protein as well as a ~70 kDal protein. A ~75 kDal protein band was also seen, albeit as a very low signal. An examination of control urine demonstrated very low levels of the 52 and 70 kDal proteins with no visible 75 kDal signal. Background signal from secondary antibody alone was negligible. GSC 1242 Immunostaining of kidney tissue from animals dosed at 0 and 500 mg/kg D-serine confirmed that GSC protein is strongly induced in the kidney after dosing. To examine the GSC response to renal glomerular damage rather than the proximal tubule injury, urine samples from a rat study using puromycin were examined. In this nephrotoxin model it was seen that again a minimal baseline level of GSC is present at pre-dose in the urine. The response of GSC in this nephrotoxin model significantly increases at 96 hours post-dose, and is seen at high levels up to 168 hours post-dose. These very early pre-validation studies on rat group specific component indicate that it is present in the urine at low levels which dramatically increase upon exposure to both puromycin and D-serine, an indication that GSC response is not localized to proximal tubule damage.

3.2.2 GSC Introduction

Group Specific Component, also called the Vitamin D Binding Protein (VDBP) is a 52 kDal (rat) monomeric glycoprotein found in the blood. GSC is synthesized in the liver in an estrogen dependent manner (Aarskog et al., 1983). GSC has been shown to be widely distributed in the tissues, and this protein or its Vitamin D complex is removed from the blood by many tissues, including the kidney, liver, muscle, heart, lung, intestine and bone. Multiple proteases control GSC catabolism, and usually only small peptide remnants of GSC are found in plasma and urine (Haddad et al., 1981). Liver disease and nephritic conditions usually exhibit low GSC levels in the blood, either due to a limitation in synthesis or due to excessive excretion of the protein (Cooke et al., 1989). GSC levels are not affected by seasonal variations or age,

remaining at a fairly stable plasma concentration (Bouillon et al., 1981), ideal considerations for its use as a urinary marker.

As a member of the albumin, a-fetoprotein and a-albumin/afamin gene family, GSC contains structural similarities to human serum albumin (Otterbein et al., 2002, Ray et al., 1991). As it is found in the blood in titers in excess of that required for Vitamin D transport alone, it is thought to have several functions (Constans, 1992). Additional studies have indicated that this protein also participates in fatty acid transport, macrophage activation, and chemotaxis. In addition to its role in Vitamin D transport, GSC binds actin found in the blood due to cellular injury, particularly mechanical damage to muscle. GSC has been shown to bind actin at a fairly high affinity rate $K_d = 10^{-9}M$ (Haddad, 1984) with the binding domain located at residues 350 to 403 (Haddad, 1992).

Interestingly, this protein exists as a highly polymorphic serum protein, and studies have linked GSC variants to osteoporosis, Graves' disease, Hashimoto's thyroiditis, diabetes, COPD, AIDS, multiple sclerosis, sarcoidosis, and rheumatic fever (Speeckaert et al., 2006). Studies examining nephrotic syndrome tissue, end-stage renal disease tissue, and controls indicated that expression of GSC, as well as α_1-microglobulin and β_2-microglobulin, increased only in the straight tubular segment of the cystinotic samples (Wilmer et al., 2008). It is thought that as GSC-VitD complex is endocytosed into the proximal tubules where the Vitamin D is activated, thus increased levels of urinary GSC may be linked to tubular injury (Uchida et al., 2007). GSC has also been seen in the urine in response to uranium nephrotoxicity (Malard et al., 2009) as well as in the urine of Cd-exposed humans (Uchida et al., 2007). At this time, there are no published studies to localize GSC release within the kidney segments or data to indicate if GSC urinary release upon renal damage is indicative of proximal tubular damage.

3.2.3 GSC Methods, Assumptions, and Procedures

Urine and Serum Collection

More detailed descriptions of the animal studies are described in *Biomarkers of Exposure to Toxic Substances, Vol 1* (DelRaso et al., Sep 2009). However, for clarity of this section, we include brief descriptions of each study.

D-serine Study 1

Male Fischer 344 rats were dosed with 5, 20, 50, 200 or 500 mg/kg D-serine in saline, whereas control animals were dosed with saline alone. Five animals were included in each dosing group with the exception of the 200 mg/kg group in which only four animals were included. Urine was collected in metabolic cages from animals prior to dosing and at 24, 48, 72 and 96 hours post-dose . One percent sodium azide, a bacteriostatic preservative, was added to each sample, and urine was frozen at -20°C within 1 hour of end point collection.

D-serine Study 2U

Male Fischer 344 rats were dosed with 200 or 500 mg/kg D-serine in saline, whereas control animals were dosed with saline alone. Five animals were included in each dosing group

with the exception of the control group in which only four animals were included. Urine was collected from animals prior to dosing and at 12-hour intervals up to 168 hours post-dose. Protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) equal to 1/100 of the urine volume was added, and urine was frozen immediately at -20°C before being moved into a -80°C freezer within 1-7 days.

D-serine Study 2S

Male Fischer 344 rats were dosed with 200 or 500 mg/kg D-serine in saline, whereas control animals were dosed with saline alone. Ten animals were included in each dosing group. Five of the ten animals were sacrificed 12 hours post-dose. The remaining five animals were sacrificed 24 hours post-dose. Blood was collected from the inferior vena cava.

Puromycin Study

Male Fischer 344 rats were dosed intraperitoneally with control (0), 5, 25, 75, 150 or 300 mg/kg puromycin in 0.9% saline. Five animals were included in each dosing group with the exception of the control group in which only four animals were included. Urine was collected in metabolic cages from animals prior to dosing and at 24, 48, 72, 96, 120, 144 and 168 hours post-dosing. Four of the five animals in dosing group 300 mg/kg died during the study, and, therefore, the sample set at that dose is incomplete. One percent sodium azide, a bacteriostatic preservative, was added to each sample, and urine was frozen at -20°C within 1 hour of end point collection.

Polyclonal Antibody Design to Rat GSC

Group Specific Component Access No. Q68FY4 was verified by the RHPB Proteomics group as a potential marker using D-serine rat urine from Study 1 (Figure 85). Two regions (marked in red) were selected based on their lack of homology to other sequences as well as for their ability to initiate a strong immuno-reactive response in the animal (predicted outer protein structure location, charged amino acids).

```
LOCUS       NP_036696                    476 aa
        DEFINITION  group specific component [Rattus norvegicus].
        ACCESSION   NP_036696
        VERSION     NP_036696.1  GI:6978879
        DBSOURCE    REFSEQ: accession NM_012564.1
        SOURCE      Rattus norvegicus (Norway rat)
```

GSC 1239

```
  1    MKRVLVLLLA LAFGHALERG RDYEKDKVCQ ELSTLGKDDF RSLSLILYSR
 51    KFPSSTFEQV SQLVKEVVSL TEECCAEGAD PNCYDTRTSE LSIKSCESDA
101    PFPVHPGTSE CCTKEGLERK LCMAALSHQP QEFPAYVEPT NDEICEAFRK
151    DPKGFADQFL FEYSSNYGQA PLPLLVGYTK SYLSMVGSCC TSAKPTVCFL
201    KERLQMKQLL LLTTMSNRVC SQYAAYGKEK SRMSHLIKLA QKVPTANLED
251    VLPLAEDLTE ILSRCCKSTS EDCMARELPE HTLKICGNLS KKNSKFEECC
301    YETTPMGIFM CSYFMPTAEP LQLPAIKLPT SKDLCGQSAT QAMDQYTFEL
351    SRRTQVPEVF LSKVLDTTLK TLRECCDTQD SVSCFSTQSP LMKRQLTSFI
401    EKGQEMCADY SENTFTEYKK KLAERLRTKM PNASPEELAD MVAKHSDFAS
451    KCCSINSPPR YCSSQIDAEM RDILQS
```

GSC 1240

Figure 85: Peptide Antigen sequences of Group Specific Component Rat Polyclonal antibodies

Polyclonal Antibody Development to Rat GSC

Peptides were generated by New England Peptide, LLC. Purity was tested by HPLC and was≥ 85% by percent area on the standard HPLC gradient. MALDI-TOF DE mass spectral analysis was performed to ensure peptide mass was within 0.1% of the exact molecular weight. The peptides were used to immunize two new Zealand White rabbits (specific pathogen free). Antigen preparations included the use of adjuvants. Complete Freund's Adjuvant (CFA) was used in the first injection only. Subsequent booster immunizations, on day 14 and 28 after initial injection, used Incomplete Freund's Adjuvant (IFA). One week after the last immunization, sera from the first production bleed were collected (Table 32). A second production bleed was performed 4 days later. An affinity purification column was synthesized using 4 mg of peptide. A minimum of 5 mg of specific antibody were purified from 40-50 mL of antiserum. Purified antibody was supplied in PBS, pH 7.4. Peptide was supplied as a lyophilized powder containing traces of trifluoroacetic acid. Purified polyclonal antibodies from two rabbits (F3117 and F31118) against peptide 1239 were combined and designated GSC 1239, while the purified polyclonal antibodies from antigen peptide 1242 (rabbits F3123 and F3124) were designated GSC 1242.

Table 32: GSC peptide antigens and the final yields of affinity purified polyclonal antibodies

Project #	Protein	A.A. Region	Lot #	Conc. (mg/mL)	Yield (mg)	Vol. (mL)
1239	GSC	49-61	PN9188 Ac-SRKFPSSTFEQVSC-amide	3.63	19.6	5.40
1242	GSC	436-451	1515-1/3 Ac-CKEELADMVAKHSDFASK-amide	4.58	25.19	5.50

Western Analysis of GSC Expression

Western Blots were used to probe the urine samples in order to visualize changes in GSC expression. Equal amounts of total protein (10 ug) were denatured for 5 minutes at 85-100 °C. The samples, control (0 mg/kg, 24 hrs) and Precision Plus Protein Kaleidoscope standard were separated by gel electrophoresis at 100-150 V for approximately 1 hr using Bio-Rad Ready Gels (Cat. No. 161-1155 and 161-1173). The proteins were transferred onto Bio-Rad PVDF membrane using a Hoefer semi-dry blotter at 200 mA for 1 hour. The PVDF membrane was blocked for 30 minutes to 1 hour using 1% Blot-Qualified BSA (ProtoBlot® II AP System with Stabilized Substrate, Promega) and incubated with primary polyclonal antibody for 1-2 hours. Following a 30 minute to 1 hour incubation with goat anti-rabbit IgG AP conjugate, Western Blue® Stabilized AP Substrate was applied to the blot for color development.

3.2.4 GSC Results and Discussions

Polyclonal Acquisition and Initial Characterization

The final affinity purification step of each GSC polyclonal combined the two animals used per antigen, so each polyclonal is the product of the antigenic response of two animals, not one. Both GSC 1239 and GSC 1242 polyclonal antibodies were examined using urine which was predicted to contain a maximum level of protein expression in response to the nephrotoxin D-serine based on prior pre-validation work (500 mg/kg, 24 hour time point). To test for non-specific staining of the blot by the secondary antibody, Western blots were prepared which used only the secondary antibody (Figure 86). The secondary antibody was used against a control urine sample (266-20mg/kg-pre) and a high-dose urine sample (353-500mg/kg-24h). Little background was seen with the secondary AP antibody alone, whereas a very strong signal was achieved using both the GSC polyclonal as the primary plus the secondary antibody (data not shown) suggesting that non-specific binding of the secondary antibody is not responsible for the observed bands.
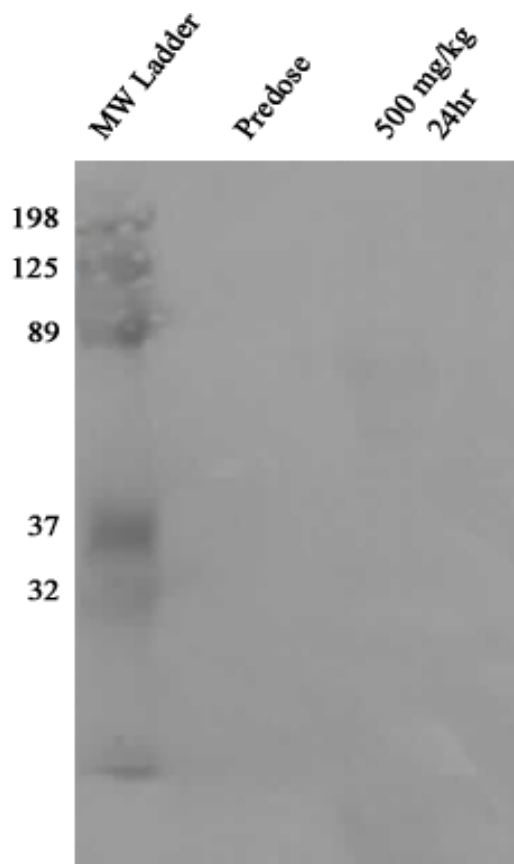
Figure 86: Western Blot Examining Potential Secondary Antibody Background
Analysis using secondary antibody without primary antibody

To further characterize the developed polyclonal antibodies, the GSC 1242 antibody was used against a control urine sample (0mg/kg, pre-dose) and a high D-serine dose urine sample (500mg/kg, 24 hour post-dose). Three concentrations of primary antibody were used: 0.25, 0.5 and 1 ug/mL. Approximately 52 kDal and 70 kDal protein bands were observed in the control sample and the high dose sample at all three concentrations of primary antibody. Analysis suggested that 0.5 ug/mL is the appropriate primary antibody concentration for Western analyses.

GSC Urine Expression Profile in a Puromycin Nephrotoxin Model

The nephrotoxin D-serine primarily targets the proximal tubules of the kidney, and initial results indicate that GSC is released into the urine (Figure 87), although additional analyses will be conducted to determine the response time in this model. However, in order to determine if the GSC response is specific to proximal damage or can be seen in response to decrement in other kidney regions, additional urine samples were tested which were produced in rats dosed with puromycin at levels thought to induce subclinical kidney damage. Puromycin has been shown to induce glomerular injury to the kidney (Sections 1.4.1 and 1.4.2, DelRaso et al., Sep 2009), and therefore would be a good model to examine the regional specificity of the biomarker response.

Figure 87: Western Blot Analysis of rat urine using GSC 1242

Western blots using polyclonal GSC 1242 and urine collected from two different animals (318 and 317) dosed with 150 mg/kg puromycin were examined for the presence of the 52 kDal GSC band (Figure 88). Two different animals were used due to limited sample availability. Urine from a known GSC positive sample from the D-serine study was used as a positive control. As before, a strong 52 kDal signal can be seen (Figure 88, red arrow) although at time points >96 hours. In addition, concomitant increases in the 70 kDal protein were seen (Figure 88, blue arrow). Use of puromycin as the nephrotoxicant seemed to require a longer time period to stimulate the increase of GSC protein levels in the urine. The longer time period needed to induce expression changes in urine biomarkers, versus that seen in the D-serine model, was also seen during proteomic analysis of the puromycin-dosed animal urine (Dr Pavel Shiyanov, personal communication). Puromycin selectively induces nephrosis in the glomerular visceral epithelial cells and has been shown to induce selective proteinuria 4 to 5 days after a single intravenous or intraperitoneal injection, although epithelial cell damage can be detected 24 hours post-dose using electron microscopy (Lawrence et al., 1983; Bhuyan et al., 1980).

As shown, initial data indicate that the GSC protein is seen using two nephrotoxins targeting different regions of the kidney. It is plausible that GSC is indicative of tissue damage at the epithelial cells in the glomerulus as well as the proximal tubules. Analysis in a third toxin model targeting the distal tubules would provide additional data as to the utility of this marker against different kidney segment injury. These initial results may indicate that GSC is a general biomarker inclusive of all kidney epithelial cell injury rather than indicative of damage to a specific kidney region.

161

Figure 88: Western Blot Analysis of rat urine from Puromycin Study using polyclonal GSC 1242

Urine from a 200 mg/kg D-serine dosed animal taken at 24 hour post-dose was used as the positive control

Immunostain Verification of GSC Expression in the Kidney

In order to verify that GSC protein changes are indeed seen in the kidney, immunostaining of sagittal and transverse kidney sections was performed in conjunction with the Proteomics group using polyclonal GSC 1242 (Section 3.1, Shiyanov et al., 2009). In both the sagittal and transverse sections, a strong GSC signal can be seen in the dosed kidney (500 mg/kg, 12 hours) versus control (0 mg/kg, 12 hours). While there is a basal level of GSC expression in control sections (Figure 89, A and C) as supported in the literature, the GSC signal is ***clearly*** significantly increased in dosed sections (Figure 89, D and B). This data provides significant support for GSC as a urinary biomarker of kidney injury as GSC expression changes can be seen at the kidney tissue level.

Both Western analyses and immunostaining data on GSC increases in the urine, confirms that the LC/MS semi-quantitative data reported in Shiyanov et al., 2009, as well the identification of GSC, is accurate and not artifactual in nature.
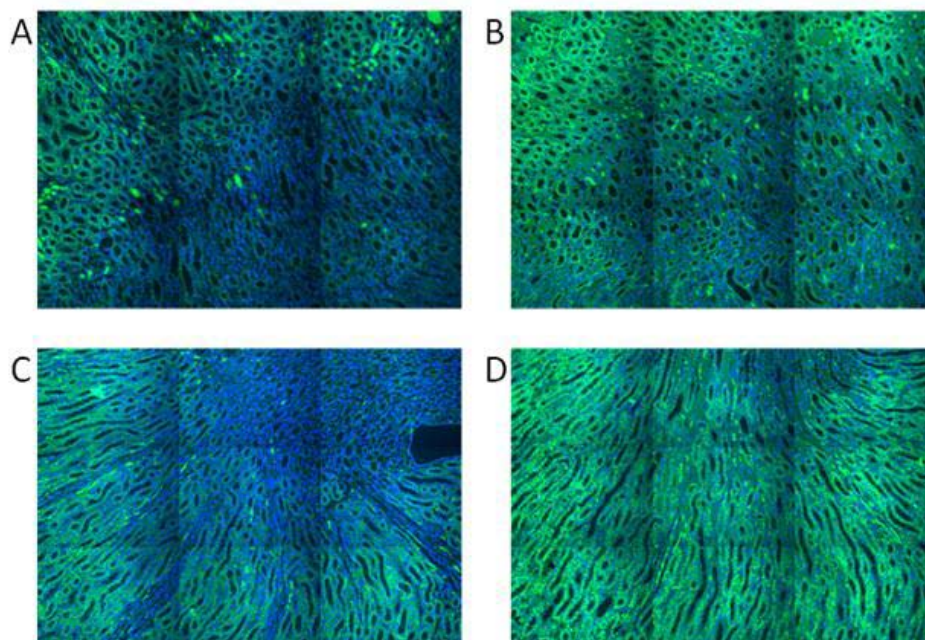
Figure 89: Immunostaining for GSC in D-serine dosed and Control kidney
GSC 1242 polyclonal antibody was used for staining.
A and B – sagittal sections of kidney.
C and D – transverse sections of kidney.
A and C are controls at 12h. B and D are kidneys from animals after
500mg/kg D-serine uptake, 12h.

4. CONCLUSIONS

4.1 Journal-based Biomarker Analyses

4.1.1 Protein Marker Pre-validation Summation

During the course of this study, 16 candidate proteins were listed as possible biomarkers indicative of early subclinical renal damage in response to low level toxin exposures. Of the 16 proteins examined, 11 were further evaluated and the biomarker response profiles determined. In the remaining 5 assays, two ELISAs were validated for quantitation of the biomarker in rat urine, but the initial tests indicated that the protein levels did not change or what change was seen was not significant (IL-18, proANP). For the last three markers, it was seen that the corresponding ELISA tests were not capable of duplicating dilutional linearity and therefore could not successfully quantitate the biomarker in rat urine (ET-1, IL-6, Fibronectin). In these three cases, the marker expression may very well modulate upon renal injury, but the assay was unable to provide valid data to conduct proper evaluations. For pre-validation of ET-1, IL-6, and Fibronectin, full evaluation will have to await development of sensitive ELISAs which have minimal urine interference and which are capable of recognizing the rat homolog.

4.1.2 ELISA Test Validations in Urine

Many of the commercially available tests for the protein markers were not validated for either use with rat samples, or were not validated for use in urine. In order to utilize these assays in the rat nephrotoxin biomarker pre-validation, it was necessary to validate the ELISA using our animal model and our test matrix. These studies were not *biomarker* validations, but additional tests conducted on the ELISA to determine if the kit could perform under conditions for which it was not validated nor marketed. Most commercial vendors were kind enough to provide free or greatly discounted ELISA plates for most of these studies. Assay validations were conducted on ELISAs for Clusterin, TIMP1, RBP4, ET-1, IL-18, proANP, IL-6, HO-1, and fibronectin. The ET-1 and proANP ELISAs were marketed as human test kits which needed validation using rat proteins, while Clusterin, RBP4, IL-18, IL-6, fibronectin, TIMP1 and HO-1 required test matrix validation. As discussed in the previous sections, assays to ET-1, fibronectin, and IL-6 did not validate under these new conditions and, therefore, could not be further analyzed. ELISAs that were validated by this lab for use for testing in rat urine included Clusterin, RBP4, HO-1, and TIMP1. The level and standard of RHPB assay validations met or exceeded the levels found in manufacturer testing.

4.1.3 Serum Biomarker Response to Subclinical Renal Injury

In order to determine whether changes in the protein expression profile can be detected in blood prior to urine, a subset of the analyzed putative biomarkers were quantitated in serum samples as well as in urine using ELISA. Due to the availability of ELISAs capable of analyte quantitation in a serum test matrix, as well as funding limitations, serum testing was conducted in only a subset of the biomarker list. In addition to assay limitations, testing was also limited to serum samples collected during Study 2S. Longer daily blood draws and sample collection in Study 1 was limited to blood draws of 300 uL or less due to IACUC regulations. Therefore, the majority of these samples were entirely consumed during Proteomics analyses. It is unfortunate

that a larger animal number could have been used to increase the availability of serum samples for full serum profiling, or that a secondary animal study conducted to produce additional serum samples from which to pre-validate markers. Even so, samples from 12 and 24 hour post-dose from Study 2S were analyzed for responsiveness to D-serine exposure to provide some data as to the potential serum responses of a subset of markers.

An examination of six proteins in the serum of D-serine injected rats indicated that Clusterin and RBP4 provided a significant and fast response to the nephrotoxin within 12 hours of exposure (Figure 90). TIMP1, β_2-microglobulin, and αGST exhibited smaller increases at 12 hours post-dose. This study indicates that of the six biomarkers examined in blood samples from the D-serine nephrotoxin rat model, Clusterin and RPB4 are the most responsive. Urine studies, discussed below, also indicated that clusterin is one of the more responsive markers tested (serum was not tested for KIM1). A follow up study replicating Study 1 with daily blood draws would provide additional data as to the marker profile and length of response. Samples obtained from other nephrotoxin models would also provide some data as to the renal segment specificity.
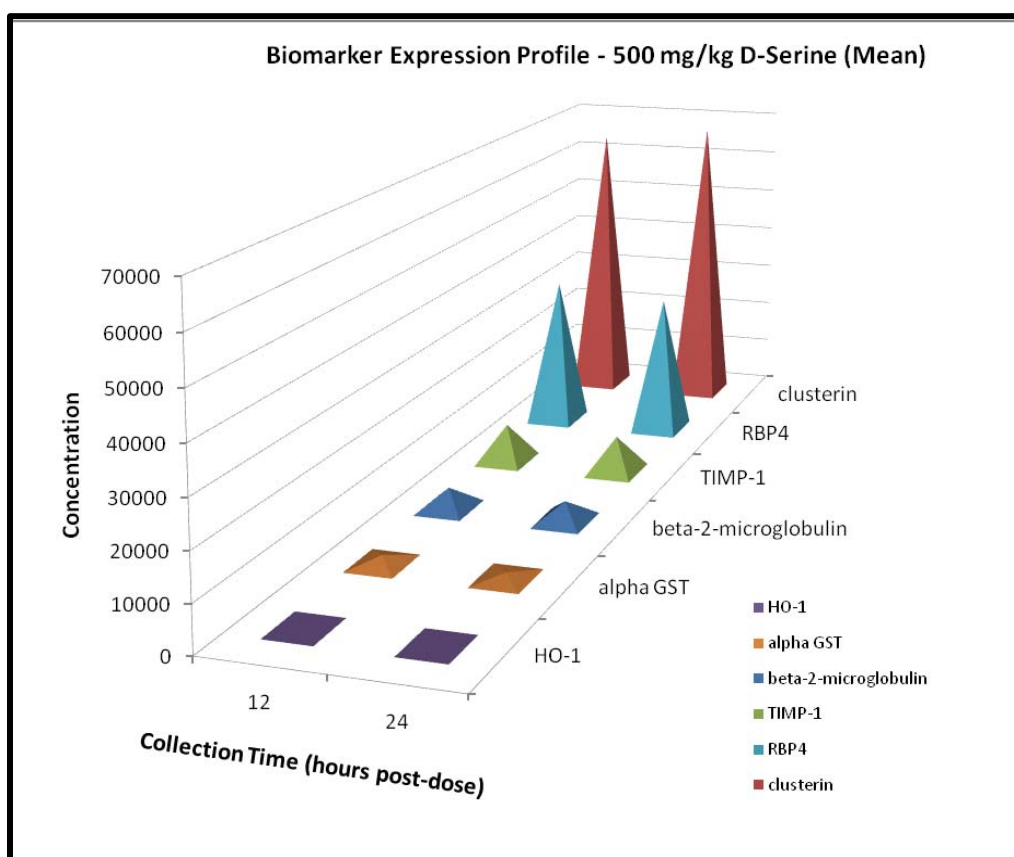
Figure 90: Serum Biomarker response in a D-serine nephrotoxin model

4.1.4 Nephrotoxin Responsiveness at the Gene Level

Once the final list of biomarkers was developed, these genes were also examined using expression data collected during this study by the Genomics group. In these analyses, RNA was

isolated from control and dosed kidneys and used to probe Affymetrix Gene chips (Chan et al., 2009). The urinary protein expression profiles were only obtained for D-serine exposures with some preliminary examination in a puromycin model. However, an examination of the expression levels of ten genes generated across a span of nephrotoxins (D-serine, puromycin, amphotericin B, and 2-bromoethylamine) yielded interesting insights as to the responsiveness of renal tissue to different toxins (Figure 91). As can be seen, a subset of well characterized biomarkers (KIM-1, Clusterin, TIMP1, Osteopontin) exhibit increases in all four nephrotoxin exposures. Fibronectin also demonstrated fold increases in all toxins tested, but as previously discussed, the ELISA was not able to quantitatively analyze the protein marker. Two other well studied biomarkers to kidney injury, cystatin C and NGAL demonstrated fold increases in the D-serine and puromycin study, or in the D-serine study alone, respectively. β2M also demonstrated a very small fold increase in the D-serine study. An examination of the responsiveness of each biomarker to the nephrotoxin array indicates that a number of the markers express in respond to three or more toxins (Table 33). Clusterin, KIM1, OSN, RBP4, TIMP1, and Fibronectin all demonstrate gene expression increases after the kidney is subjected to nephrotoxins which targeted different kidney segments. Since whole kidney was used in the microarray analysis, the possibility that these nephrotoxins did indeed induce region-specific differential gene expression cannot be ruled out.
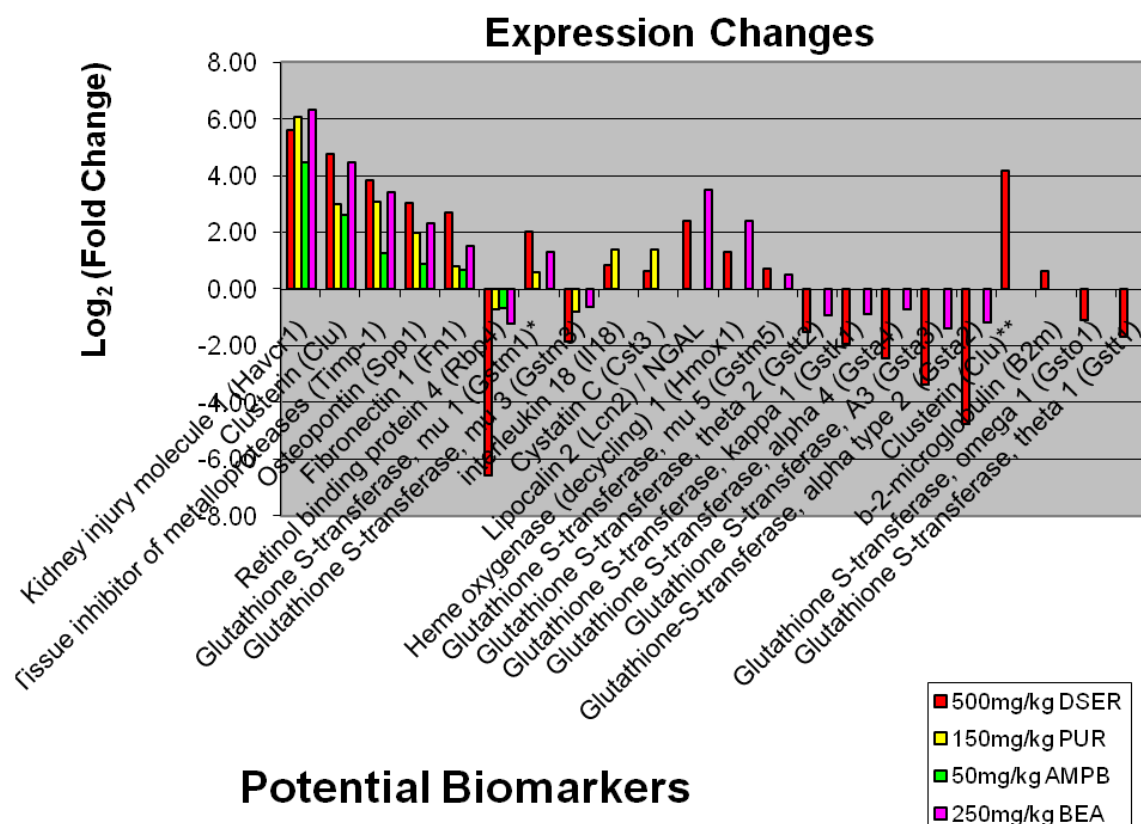
Figure 91: Differential Gene Expression in control vs. dosed kidney for Pre-validated Journal-based Biomarker set

Table 33: Summary of Kidney RNA Expression levels of Journal-based biomarkers

D-Serine	Puromycin	Amphotericin B	2-Bromoethylamine	Count
Clusterin (Clu)	Clusterin (Clu)	Clusterin (Clu)	Clusterin (Clu)	4
Kidney injury molecule 1 (Havcr1)	Kidney injury molecule 1 (Havcr1)	Kidney injury molecule 1 (Havcr1)	Kidney injury molecule 1 (Havcr1)	4
Fibronectin 1 (Fn1)	Fibronectin 1 (Fn1)	Fibronectin 1 (Fn1)	Fibronectin 1 (Fn1)	4
Osteopontin (Spp1)	Osteopontin (Spp1)	Osteopontin (Spp1)	Osteopontin (Spp1)	4
Retinol binding protein 4 (Rbp4)	Retinol binding protein 4 (Rbp4)	Retinol binding protein 4 (Rbp4)	Retinol binding protein 4 (Rbp4)	4
Tissue inhibitor of metalloproteases (Timp-1)	Tissue inhibitor of metalloproteases (Timp-1)	Tissue inhibitor of metalloproteases (Timp-1)	Tissue inhibitor of metalloproteases (Timp-1)	4
Glutathione S-transferase, mu 1 (Gstm1)*	Glutathione S-transferase, mu 1 (Gstm1)*		Glutathione S-transferase, mu 1 (Gstm1)*	3
Glutathione S-transferase, mu 3 (Gstm3)	Glutathione S-transferase, mu 3 (Gstm3)		Glutathione S-transferase, mu 3 (Gstm3)	3
Cystatin C (Cst3)	Cystatin C (Cst3)			2
Interleukin 18 (Il18)	Interleukin 18 (Il18)			2
Glutathione S-transferase, A3 (Gsta3)			Glutathione S-transferase, A3 (Gsta3)	2
Glutathione S-transferase, alpha 4 (Gsta4)			Glutathione S-transferase, alpha 4 (Gsta4)	2
Glutathione S-transferase, kappa 1 (Gstk1)			Glutathione S-transferase, kappa 1 (Gstk1)	2
Glutathione S-transferase, mu 5 (Gstm5)			Glutathione S-transferase, mu 5 (Gstm5)	2
Glutathione S-transferase, theta 2 (Gstt2)			Glutathione S-transferase, theta 2 (Gstt2)	2
Glutathione-S-transferase, alpha type 2 (Gsta2)			Glutathione-S-transferase, alpha type 2 (Gsta2)	2
Heme oxygenase (decycling) 1 (Hmox1)			Heme oxygenase (decycling) 1 (Hmox1)	2
Lipocalin 2 (Lcn2) / NGAL			Lipocalin 2 (Lcn2) / NGAL	2
β-2-microglobulin (B2m)				1
Clusterin (Clu)**				1
Glutathione S-transferase, omega 1 (Gsto1)				1
Glutathione S-transferase, theta 1 (Gstt1)				1
*Also know as GST Yb1				
**Second probe set				

167

In examining renal injury biomarker responses, it is of interest to determine if such increases are due to stimulation in gene expression, increases in secretion from damaged cells, or both. In examining the gene expression data, it is clear that Kim-1 is one of the most responsive biomarkers at the RNA level, also seen in other studies (Ichimura et al., 2004) in which RNA levels increase in the proximal tubule epithelial cells in the post-ischemic rodent and human kidney. At the level of protein secretion into the urine, however, Kim-1 has a relatively slow response (120-144 hours post-dose) in the D-serine model. Interestingly β2M RNA increases were only seen in the D-serine model and at moderate fold increases, while as a urinary marker this protein increases at significant levels at 12 hour post-dose. This indicates that the increases seen in urinary β2M expression are most likely due not to an increase in protein synthesis, but to an increase in secretion due to injury and decrement in glomerular filtration.

Interestingly, RBP4 demonstrated a marked decrease in gene expression upon exposure to all four nephrotoxins (Figure 91), the only ELISA tested marker to demonstrate significantly lower gene expression while exhibiting increased urinary levels upon D-serine exposure (Fig. 27). The opposing dynamics of RPB4 RNA versus protein expression is unseen in any other RBP4 biomarker publications to date, and implies that increases are due to increased secretion or cellular dump mechanisms.

4.1.5 Dose Responsiveness at the Gene Level

If each dose is examined for RNA increase (or decrease) among the journal-based gene set, patterns emerge on the utility of different markers (Table 34, Table 35). Again, Kim-1 is seen as universally responsive to all nephrotoxins tested and at lower doses. Clusterin, TIMP1, and OPN are likewise responsive, although the fold differences are not as high as those found with Kim1. NGAL, another widely examined urine and serum renal injury marker, does not exhibit as robust and generalized response as expected from published research. Therefore at a gene expression level in the nephrotoxin-dosed kidney, Kim-1 is markedly superior in response and sensitivity.

The histopathology data confirmed that, within the doses used, visible cellular damage occurred at the higher doses selected. In the case of D-serine exposures, injury was identified only in the 200 mg/kg dose group and higher (Table 36). Therefore, the lack of significant differences at 5, 20, and 50 mg/kg may be explained in that the dose levels were too low to induce detectable changes. In amphotericin B (AMPB) exposures, the only fold increases in this biomarker subset were seen in the 50 mg/kg dose group (Table 39), while expression in animals injected with puromycin exhibited RNA increases only in the highest dose group of 150 mg/kg (Table 38). 2-bromoethylamine (BEA) changes were seen even at the lowest dose of 1 mg/kg (Table 37). Very little expressional change was seen in the hippuric Acid (HPA) nephrotoxin model, indicating that the dose selections were too low to induce low level injury (Table 40). Thus, at least for D-serine, and puromycin, the doses reflected accurately modeled low level renal injury. Lower doses of BEA (< 1 mg/kg) may have provided additional data on boarder-line renal injury. HPA doses were too low to produce the required renal injury, and AMPB did not prove effective due to issues discussed in DelRaso et al., Sep 2009.

Table 34: Differential Gene Expression in control vs. dosed kidney for Pre-validated Journal-based Biomarker in four nephrotoxin studies

Fold Change				
Description	500mg/kg DSER	150mg/kg PUR	50mg/kg AMPB	250mg/kg BEA
Kidney injury molecule 1 (Havcr1)	48.13	66.55	21.81	79.53
Clusterin (Clu)	27.38	7.94	6.14	21.84
Tissue inhibitor of metalloproteases (Timp-1)	14.23	8.43	2.38	10.68
Osteopontin (Spp1)	8.18	3.92	1.87	5.00
Fibronectin 1 (Fn1)	6.44	1.75	1.61	2.83
Retinol binding protein 4 (Rbp4)	0.01	0.61	0.64	0.43
Glutathione S-transferase, mu 1 (Gstm1)*	4.11	1.49		2.50
Glutathione S-transferase, mu 3 (Gstm3)	0.28	0.58		0.65
interleukin 18 (Il18)	1.81	2.63		
Cystatin C (Cst3)	1.54	2.65		
Lipocalin 2 (Lcn2) / NGAL	5.25			11.17
Heme oxygenase (decycling) 1 (Hmox1)	2.46			5.33
Glutathione S-transferase, mu 5 (Gstm5)	1.65			1.40
Glutathione S-transferase, theta 2 (Gstt2)	0.35			0.53
Glutathione S-transferase, kappa 1 (Gstk1)	0.26			0.54
Glutathione S-transferase, alpha 4 (Gsta4)	0.19			0.60
Glutathione S-transferase, A3 (Gsta3)	0.10			0.38
Glutathione-S-transferase, alpha type 2 (Gsta2)	0.04			0.44
Clusterin (Clu)**	18.23			
β-2-microglobulin (B2m)	1.56			
Glutathione S-transferase, omega 1 (Gsto1)	0.47			
Glutathione S-transferase, theta 1 (Gstt1)	0.31			

Table 35: Log$_2$ (Fold Change) Differential Gene Expression in control vs. dosed kidney for Pre-validated Journal-based Biomarker in four nephrotoxin studies

Log$_2$(Fold Change)				
Description	500mg/kg DSER	150mg/kg PUR	50mg/kg AMPB	250mg/kg BEA
Kidney injury molecule 1 (Havcr1)	5.59	6.06	4.45	6.31
Clusterin (Clu)	4.78	2.99	2.62	4.45
Tissue inhibitor of metalloproteases (Timp-1)	3.83	3.07	1.25	3.42
Osteopontin (Spp1)	3.03	1.97	0.90	2.32
Fibronectin 1 (Fn1)	2.69	0.81	0.69	1.50
Retinol binding protein 4 (Rbp4)	-6.57	-0.72	-0.65	-1.21
Glutathione S-transferase, mu 1 (Gstm1)*	2.04	0.57		1.32
Glutathione S-transferase, mu 3 (Gstm3)	-1.84	-0.80		-0.63
interleukin 18 (Il18)	0.86	1.39		
Cystatin C (Cst3)	0.63	1.41		
Lipocalin 2 (Lcn2) / NGAL	2.39			3.48
Heme oxygenase (decycling) 1 (Hmox1)	1.30			2.41
Glutathione S-transferase, mu 5 (Gstm5)	0.73			0.49
Glutathione S-transferase, theta 2 (Gstt2)	-1.52			-0.93
Glutathione S-transferase, kappa 1 (Gstk1)	-1.95			-0.89
Glutathione S-transferase, alpha 4 (Gsta4)	-2.43			-0.74
Glutathione S-transferase, A3 (Gsta3)	-3.38			-1.39
Glutathione-S-transferase, alpha type 2 (Gsta2)	-4.74			-1.17
Clusterin (Clu)**	4.19			
β-2-microglobulin (B2m)	0.64			
Glutathione S-transferase, omega 1 (Gsto1)	-1.09			
Glutathione S-transferase, theta 1 (Gstt1)	-1.69			
*Also know as GST Yb1				
**Second probe set				

Table 36: Differential Gene Expression in control vs. dosed kidney for Pre-validated Journal-based Biomarker using low doses of D-serine (DSER)

Description	Control DSER - Average	5mg DSER - Average	20mg DSER - Average	50mg DSER - Average	200mg DSER - Average	500mg DSER - Average
Kidney injury molecule 1 (Havcr1)	1.00	2.10	0.80	0.47	37.08	48.13
Clusterin (Clu)	1.00	2.16	1.28	1.37	22.80	27.38
Clusterin (Clu)**	1.00	1.18	0.98	1.45	15.36	18.23
Tissue inhibitor of metalloproteases (Timp-1)	1.00	1.60	1.09	1.01	11.47	14.23
Osteopontin (Spp1)	1.00	1.39	0.99	1.02	6.56	8.18
Fibronectin 1 (Fn1)	1.00	1.58	1.24	1.50	6.01	6.44
Lipocalin 2 (Lcn2) / NGAL	1.00	1.19	0.98	1.25	4.16	5.25
Glutathione S-transferase, mu 1 (Gstm1)*	1.00	1.11	1.22	1.28	3.66	4.11
Heme oxygenase (decycling) 1 (Hmox1)	1.00	1.40	1.27	1.17	2.65	2.46
interleukin 18 (Il18)	1.00	0.89	1.08	1.10	1.84	1.81
Glutathione S-transferase, mu 5 (Gstm5)	1.00	1.04	1.10	1.02	1.81	1.65
β-2-microglobulin (B2m)	1.00	1.01	1.02	1.09	1.47	1.56
Cystatin C (Cst3)	1.00	1.54	1.33	1.29	1.89	1.54
Glutathione S-transferase, omega 1 (Gsto1)	1.00	0.91	1.01	0.94	0.56	0.47
Glutathione S-transferase, theta 2 (Gstt2)	1.00	0.97	1.16	1.03	0.35	0.35
Glutathione S-transferase, theta 1 (Gstt1)	1.00	0.98	0.98	0.98	0.37	0.31
Glutathione S-transferase, mu 3 (Gstm3)	1.00	0.79	0.85	0.82	0.30	0.28
Glutathione S-transferase, kappa 1 (Gstk1)	1.00	0.72	0.81	0.88	0.30	0.26
Glutathione S-transferase, alpha 4 (Gsta4)	1.00	0.89	0.95	0.86	0.24	0.19
Glutathione S-transferase, A3 (Gsta3)	1.00	0.73	0.85	0.88	0.13	0.10
Glutathione-S-transferase, alpha type 2 (Gsta2)	1.00	0.72	0.87	0.77	0.08	0.04
Retinol binding protein 4 (Rbp4)	1.00	0.73	0.74	0.72	0.03	0.01
Glutathione S-transferase, mu 2 (Gstm2)	**Change < 1.5-Fold**					
β-2-microglobulin (B2m)						
Glutathione-S-transferase, pi 1/2						
Glutathione S-transferase, mu 4 (Gstm4)	**Not Detected**					
Hepatocyte Growth Factor (Hgf)						

Remarks: data - good; dose range/duration - good

Table 37: Differential Gene Expression in control vs. dosed kidney for Pre-validated Journal-based Biomarker using low doses of 2-bromoethylamine (BEA)

Description	Control BEA - Average	1mg BEA - Average	5mg BEA - Average	15mg BEA - Average	50mg BEA - Average	150mg BEA - Average	250mg BEA - Average
Kidney injury molecule 1 (Havcr1)	1.00	3.52	21.42	24.41	1.42	42.74	79.53
Clusterin (Clu)	1.00	1.23	7.21	6.60	1.43	10.46	21.84
Lipocalin 2 (Lcn2) / NGAL	1.00	3.42	7.77	4.19	1.63	1.15	11.17
Tissue inhibitor of metalloproteases (Timp-1)	1.00	1.97	6.22	4.53	0.95	3.46	10.68
Heme oxygenase (decycling) 1 (Hmox1)	1.00	1.62	9.14	3.45	1.47	1.55	5.33
Osteopontin (Spp1)	1.00	1.62	2.39	1.81	1.02	3.84	5.00
Fibronectin 1 (Fn1)	1.00	1.11	2.20	1.68	1.09	1.74	2.83
Glutathione S-transferase, mu 1 (Gstm1)*	1.00	1.58	1.47	1.00	1.18	2.03	2.50
Glutathione S-transferase, mu 5 (Gstm5)	1.00	1.25	1.16	0.78	1.57	1.71	1.40
Glutathione S-transferase, mu 3 (Gstm3)	1.00	1.15	0.68	0.48	1.39	1.00	0.65
Glutathione S-transferase, alpha 4 (Gsta4)	1.00	1.12	0.68	0.51	0.93	0.89	0.60
Glutathione S-transferase, kappa 1 (Gstk1)	1.00	1.23	0.68	0.55	0.84	0.68	0.54
Glutathione S-transferase, theta 2 (Gstt2)	1.00	1.23	0.70	0.51	0.81	0.69	0.53
Glutathione-S-transferase, alpha type 2 (Gsta2)	1.00	1.19	0.64	0.47	0.95	0.93	0.44
Retinol binding protein 4 (Rbp4)	1.00	1.07	0.57	0.47	0.92	0.91	0.43
Glutathione S-transferase, A3 (Gsta3)	1.00	1.06	0.55	0.39	0.90	0.63	0.38
β-2-microglobulin (B2m)	**Change < 1.5-Fold**						
Glutathione-S-transferase, pi 1/2							
Glutathione S-transferase, mu 2 (Gstm2)							
Glutathione S-transferase, theta 1 (Gstt1)							
Glutathione S-transferase, omega 1 (Gsto1)							
β-2-microglobulin (B2m)							
Glutathione S-transferase, mu 4 (Gstm4)	**Not Detected**						
Hepatocyte Growth Factor (Hgf)							
Clusterin (Clu)**							

Remarks: data - questionable (due to infection of the animals); dose range/duration - good (?)

Table 38: Differential Gene Expression in control vs. dosed kidney for Pre-validated Journal-based Biomarker using low doses of puromycin (PUR)

Description	Control PUR - Average	5mg PUR - Average	25mg PUR - Average	75mg PUR - Average	150mg PUR - Average
Kidney injury molecule 1 (Havcr1)	1.00	1.94	1.43	0.99	66.55
Tissue inhibitor of metalloproteases (Timp-1)	1.00	0.95	1.08	1.61	8.43
Clusterin (Clu)	1.00	0.77	0.82	1.02	7.94
Osteopontin (Spp1)	1.00	0.72	0.72	1.10	3.92
Cystatin C (Cst3)	1.00	0.80	1.01	1.56	2.65
interleukin 18 (Il18)	1.00	0.74	0.91	1.04	2.63
Fibronectin 1 (Fn1)	1.00	0.85	0.80	1.05	1.75
Glutathione S-transferase, mu 1 (Gstm1)*	1.00	0.94	0.84	1.01	1.49
Retinol binding protein 4 (Rbp4)	1.00	0.95	0.96	0.83	0.61
Glutathione S-transferase, mu 3 (Gstm3)	1.00	0.99	0.86	0.78	0.58
β-2-microglobulin (B2m)					
Glutathione S-transferase, A3 (Gsta3)					
Glutathione-S-transferase, alpha type 2 (Gsta2)					
Glutathione S-transferase, theta 1 (Gstt1)					
Glutathione S-transferase, theta 2 (Gstt2)					
Heme oxygenase (decycling) 1 (Hmox1)		**Change < 1.5-Fold**			
Glutathione S-transferase, mu 5 (Gstm5)					
Glutathione S-transferase, mu 2 (Gstm2)					
β-2-microglobulin (B2m)					
Glutathione S-transferase, alpha 4 (Gsta4)					
Glutathione-S-transferase, pi 1/2					
Glutathione S-transferase, omega 1 (Gsto1)					
Glutathione S-transferase, kappa 1 (Gstk1)					
Glutathione S-transferase, mu 4 (Gstm4)					
Lipocalin 2 (Lcn2) / NGAL		**Not Detected**			
Hepatocyte Growth Factor (Hgf)					
Clusterin (Clu)**					
Remarks: data - good; dose range/duration - okay					

Table 39: Differential Gene Expression in control vs. dosed kidney for Pre-validated Journal-based Biomarker using low doses of amphotericin B (AMPB)

Description	Control AMPB - Average	1mg AMPB - Average	5mg AMPB - Average	10mg AMPB - Average	25mg AMPB - Average	50mg AMPB - Average
Kidney injury molecule 1 (Havcr1)	1.00	1.49	1.28	0.92	1.09	21.81
Clusterin (Clu)	1.00	1.29	1.34	1.01	1.11	6.14
Tissue inhibitor of metalloproteases (Timp-1)	1.00	1.30	1.30	1.01	1.00	2.38
Osteopontin (Spp1)	1.00	1.21	0.91	1.06	0.96	1.87
Fibronectin 1 (Fn1)	1.00	1.46	1.29	1.12	1.21	1.61
Retinol binding protein 4 (Rbp4)	1.00	0.89	1.02	0.73	0.58	0.64
β-2-microglobulin (B2m)						
Glutathione S-transferase, A3 (Gsta3)						
Glutathione-S-transferase, alpha type 2 (Gsta2)						
Glutathione S-transferase, theta 1 (Gstt1)						
Glutathione S-transferase, theta 2 (Gstt2)						
Glutathione S-transferase, mu 2 (Gstm2)						
β-2-microglobulin (B2m)		**Change < 1.5-Fold**				
Glutathione S-transferase, alpha 4 (Gsta4)						
Glutathione S-transferase, mu 1 (Gstm1)*						
Glutathione S-transferase, mu 3 (Gstm3)						
Glutathione-S-transferase, pi 1/2						
Glutathione S-transferase, omega 1 (Gsto1)						
Glutathione S-transferase, kappa 1 (Gstk1)						
Glutathione S-transferase, mu 4 (Gstm4)						
Heme oxygenase (decycling) 1 (Hmox1)						
Glutathione S-transferase, mu 5 (Gstm5)		**Not Detected**				
Lipocalin 2 (Lcn2) / NGAL						
Hepatocyte Growth Factor (Hgf)						
Clusterin (Clu)**						
Remarks: data - fair (due to the dose/duration of the treatment); dose range/duration - insufficient (the source of the problem of this data set)						

171

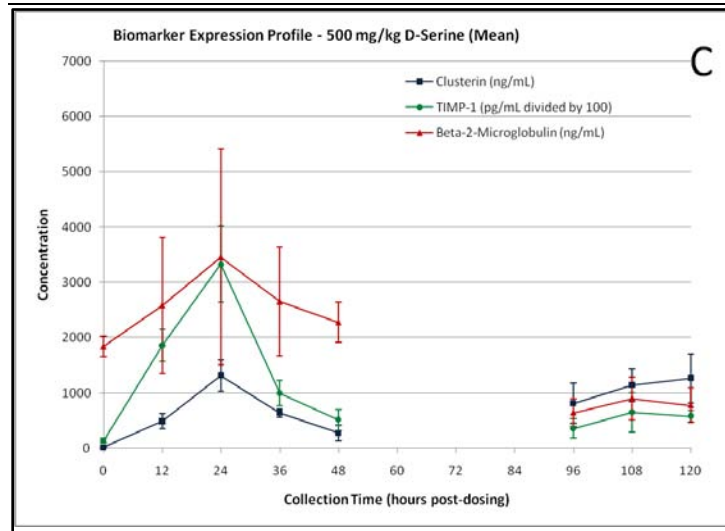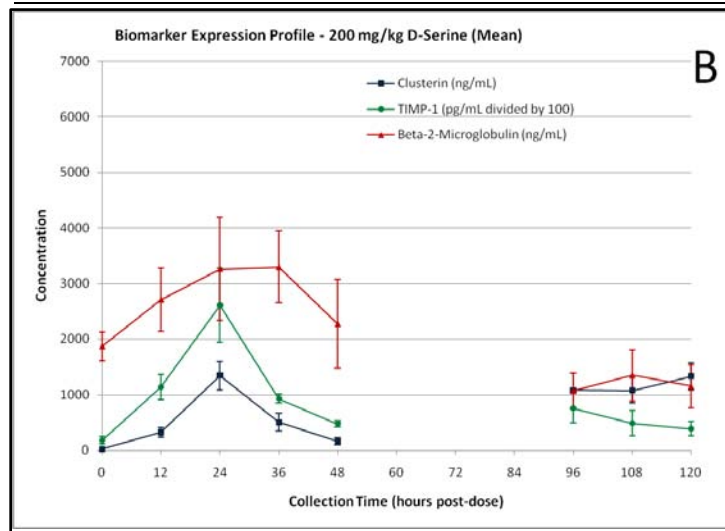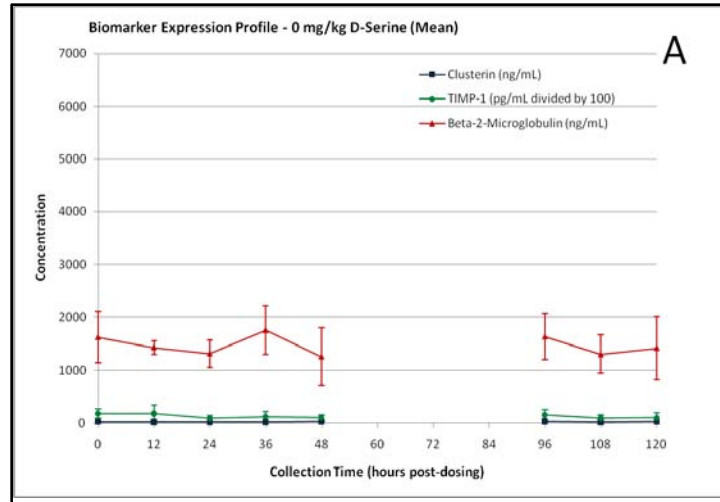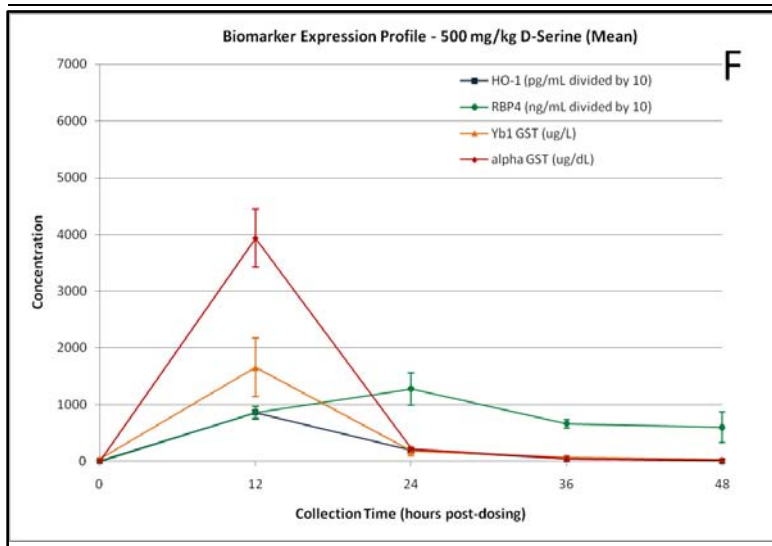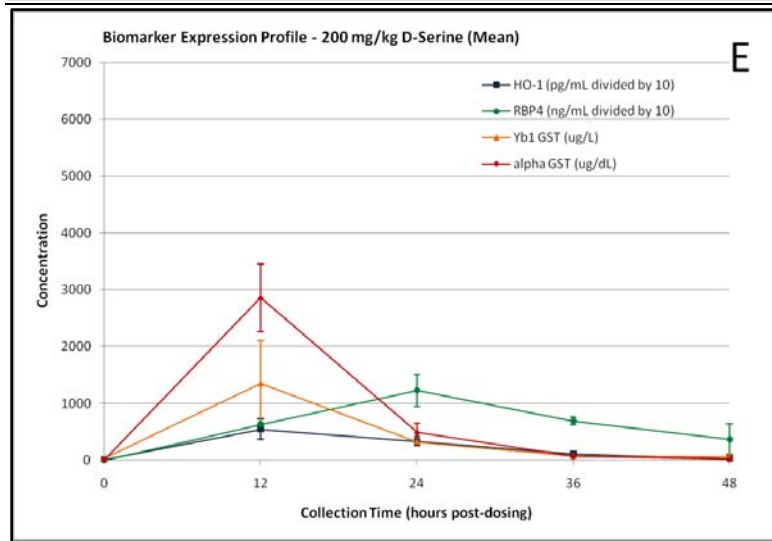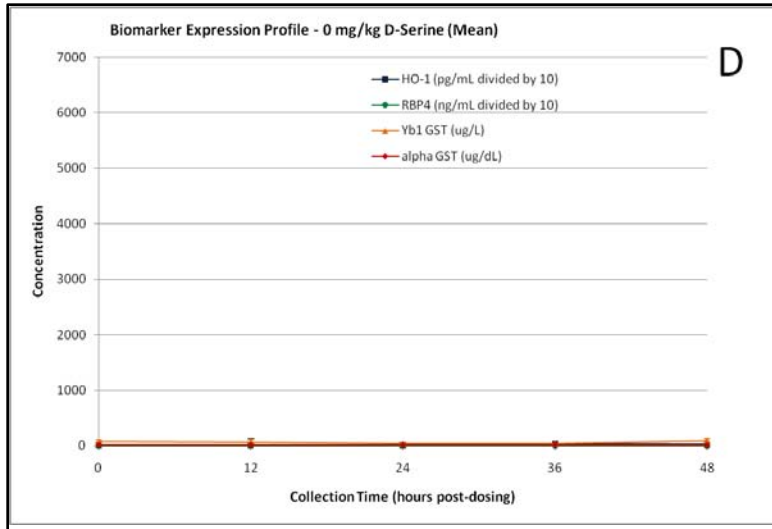**Table 40: Differential Gene Expression in control vs. dosed kidney for Pre-validated Journal-based Biomarker using low doses of hippuric acid (HPA)**

Description	Control HPA - Average	5mg HPA - Average	50mg HPA - Average	500mg HPA - Average	750mg HPA - Average	1000mg HPA - Average	1250mg HPA - Average
Glutathione S-transferase, alpha 4 (Gsta4)	1.00	1.92	1.88	2.23	0.77	0.75	0.94
Glutathione-S-transferase, alpha type 2 (Gsta2)	1.00	1.90	1.85	2.26	0.82	0.84	0.88
Glutathione S-transferase, theta 1 (Gstt1)	1.00	2.06	2.03	2.32	0.61	0.61	0.85
Glutathione-S-transferase, pi 1/2	1.00	1.65	1.62	2.21	0.78	0.66	0.84
Glutathione S-transferase, A3 (Gsta3)	1.00	1.86	1.75	2.15	0.85	0.73	0.81
Glutathione S-transferase, omega 1 (Gsto1)	1.00	1.61	1.64	2.05	0.70	0.64	0.79
Glutathione S-transferase, theta 2 (Gstt2)	1.00	2.10	1.74	1.98	0.76	0.66	0.76
Glutathione S-transferase, mu 3 (Gstm3)	1.00	2.06	2.05	2.98	0.74	0.58	0.75
Glutathione S-transferase, kappa 1 (Gstk1)	1.00	1.71	1.95	2.18	0.68	0.60	0.69
Glutathione S-transferase, mu 2 (Gstm2)	1.00	1.74	2.09	2.22	0.58	0.43	0.68
Glutathione S-transferase, mu 5 (Gstm5)	1.00	1.51	1.74	1.93	0.59	0.54	0.67
Glutathione S-transferase, mu 1 (Gstm1)*	1.00	1.75	1.77	2.10	0.53	0.52	0.54
Retinol binding protein 4 (Rbp4)	1.00	2.10	2.04	2.72	0.66	0.55	0.51
β-2-microglobulin (B2m)	1.00	0.92	1.03	1.19	0.39	0.33	0.49
Cystatin C (Cst3)	1.00	1.03	1.23	1.25	0.28	0.23	0.45
Osteopontin (Spp1)	1.00	0.54	0.68	0.73	0.26	0.19	0.45
β-2-microglobulin (B2m)	1.00	0.76	0.84	0.94	0.27	0.22	0.36
interleukin 18 (Il18)	1.00	0.47	0.45	0.56	0.24	0.17	0.29
Fibronectin 1 (Fn1)	1.00	0.38	0.59	0.73	0.19	0.11	0.21
Lipocalin 2 (Lcn2) / NGAL	1.00	0.28	0.32	0.42	0.10	0.10	0.17
Heme oxygenase (decycling) 1 (Hmox1)	1.00	0.40	0.42	0.49	0.11	0.09	0.13
Tissue inhibitor of metalloproteases (Timp-1)	1.00	0.13	0.21	0.19	0.08	0.06	0.12
Clusterin (Clu)	1.00	0.19	0.24	0.24	0.07	0.05	0.10
Kidney injury molecule 1 (Havcr1)	1.00	0.15	0.19	0.15	0.04	0.03	0.07
Glutathione S-transferase, mu 4 (Gstm4)							
Hepatocyte Growth Factor (Hgf)				**Not Detected**			
Clusterin (Clu)**							
Remarks: data - questionable; dose range/duration - questionable							
***Also know as GST Yb1**							
****Second probe set**							

4.1.6 Nephrotoxin Specificity of Urinary Protein Markers

In this study, several nephrotoxins were used to model subclinical injury at specific segments within the kidney. During the pre-validation tests in urine, the protein expression profile of each marker in urine was examined in a time course response using several doses of the known nephrotoxin D-serine (Figure 92, A-I). As with the serum samples, marker testing in other nephrotoxin models was limited to due urine sample availability. Some additional assessments were accomplished in remaining puromycin samples, but the quality of the biomarker profile was not as statistically solid as data obtained in the D-serine model.

Biomarker Expression Profile - 0 mg/kg D-Serine (Mean) — D

- HO-1 (pg/mL divided by 10)
- RBP4 (ng/mL divided by 10)
- Yb1 GST (ug/L)
- alpha GST (ug/dL)

Biomarker Expression Profile - 200 mg/kg D-Serine (Mean) — E

- HO-1 (pg/mL divided by 10)
- RBP4 (ng/mL divided by 10)
- Yb1 GST (ug/L)
- alpha GST (ug/dL)

Biomarker Expression Profile - 500 mg/kg D-Serine (Mean) — F

- HO-1 (pg/mL divided by 10)
- RBP4 (ng/mL divided by 10)
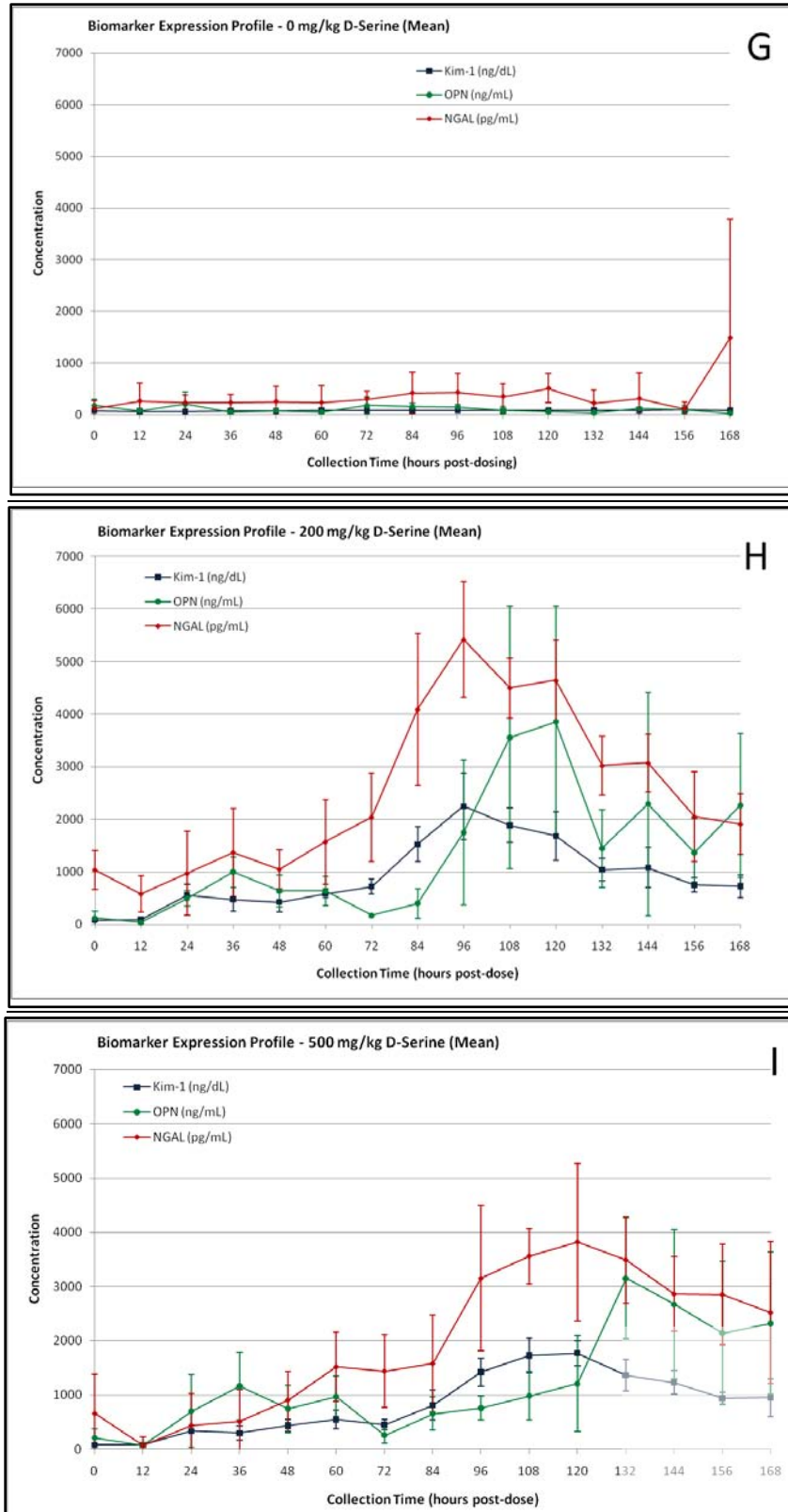- Yb1 GST (ug/L)
- alpha GST (ug/dL)

174

Figure 92: Biomarker urinary response profiles

175

4.1.7 Time/Dose Responsiveness of Urinary Protein Markers

Significant biomarker profile changes in the biomarker set were seen in urine only in the 200 and 500 mg/kg D-serine dose groups, with no significant profile changes were seen at the 5, 20 or 50 mg/kg D-serine dose groups (data not shown). As previously discussed (DelRaso et al., Sep 2009), the kidney histopathology data, clinical chemistries (TBIL, CREA, BUN, Total Protein), as well as expression data confirm the initiation of kidney damage around the 200 mg/kg D-serine dose.

Clusterin, TIMP1, and β₂-microglobulin Biomarker Profiles

In examining the β_2-microglobulin profile, it can be seen that the protein exists in the urine in significant baseline level (Figure 92, A-C). Interestingly, a biphasic response is clearly seen at 200 mg/kg with roughly equivalent peaks at 24 and 36 hours, with a distinct shift to 24 hours at the higher dose (500 mg/kg). In contrast to β2M, TIMP1 and clusterin are normally present in barely detectible levels (Figure 92, A), and shift to a maximal response at 24 hours post-dose. TIMP1 clearly exhibits dose-response expression, with peak levels at 500 mg/kg greater than that at 200 mg/kg (Figure 92 B and C). As seen, clusterin does not exhibit a dose-response, with the maximal peak levels at 24 hours post-dose roughly equivalent in 200 and 500 mg/kg urine samples (Figure 92 B and C). In all of these cases, additional testing was conducted on 96 to 120 hour post-dose samples to determine if trends seen in Study 1 indicated the presence of an additional peak or biphasic nature. Of all the markers studied, only clusterin demonstrated a biphasic nature at testing intervals between 0-120 hours post-dose.

HO-1, RBP4, Yb1GST, and α GST Biomarker Profiles

Baseline levels of all four markers are barely detectible by ELISA (Figure 92, D-F). HO-1 levels peak at 24 hours post-dose, and are seen to return to baseline within 36 hours, with little dose-response expression exhibited. Alpha GST demonstrated a very strong and fast response with a peak at 12 hours post-dose, and a fairly rapid return to baseline within 36 hours. This marker also clearly shows a dose-response levels, with higher expression at 500 mg/kg versus that seen at 200 mg/kg D-serine (Figure 92, E and F). In contrast, Yb1 GST (π GST) displays a similar response, but does *not* increase in expression with increases in nephrotoxin (Figure 92, E and F).

Kim-1, OPN, and NGAL Biomarker Profiles

The final sets of markers discussed exhibit a significantly longer response time than those discussed above. NGAL is expressed at very low baseline levels in the urine, while OPN and Kim-1 are barely detectable. Unexpectedly, the time period for maximal NGAL urinary concentration was seen to increase, not decrease, with increasing doses of D-serine (96 hour peak value versus 120 hours). The range at this time point, especially in the 500 mg/kg group, is large enough that this observation may not be valid upon closer inspection with additional testing. OPN was found to be especially sensitive to protease degradation, and handing and testing for this marker requiring especially rigorous sample handling and testing conditions. As with

NGAL, OPN responded at a much later time point that marker discussed previously but again, a peak shift to a later time point (120 versus 132 hours post-dose) was seen with increasing dosages. The Kim-1 response behaved in a similar manner, with a peak at 96 hours post-dose for 200 mg/kg increasing to 120 hours post-dose for 500 mg/kg. It is interesting that this set of three markers, all responding at later time points, exhibit an increase in the time of peak response with increased dosages, rather than the expected decrease in response time with higher exposures. In addition, the expression is not as well defined as those of TIMP1, clusterin, Yb1 GST or α GST, taking up to 3 to 4 days to reach maximal expression in the urine before a gradual decline to baseline levels. For all three markers, baseline (pre-dose) levels were not reached at 168 hours, the conclusion of sample collection in Study 2U.

In summary, several of the marker profiles, shifts in peak expression of Kim-1, NGAL, and OPN were seen between the 200 and 500 mg/kg dose groups (Figure 92, H and I). In this case, the maximal expression levels in the 200 mg/kg group were at 96 hours in NGAL and Kim-1, with OPN peak expression at 120 hours. Examination of these urinary markers at a higher dosage indicates a peak shift to 120 hours (NGAL and Kim-1) and to 132 hours for OPN. It would be expected that such a time related peak shift would *decrease,* not increase, with increased dose levels, and it not seen in most of the other biomarker profiles obtained (Figure 92, A-C, E-G). β_2-microglobulin does indicate a shift upon dose increase (Figure 92, A-C), and this shift indicates a decreased response time with increased D-serine doses. Interestingly, the expression in the 200 mg/kg dose group clearly indicates a middle range in the shift from 36 hours to 24 hours (Figure 92, B).

4.3 RHPB Subclinical Renal Damage (SCRD) Urinary Biomarker Panel Selection

To develop a panel of markers which can be used to monitor subclinical decrement due to unknown toxin exposures, it is necessary to have an understanding of both the dose response and the time response of the expression profile of each biomarker included. A marker may be slow in response (Figure 94, BM5) or fairly fast (Figure 94, BM1, BM2). In the marker panel development, it is imperative to include markers of both types to monitor both acute and chronic effects, and to estimate the time of exposure occurred. "Acute" can be defined as a biomarker response and baseline recovery in 24 hours or less (quick response, quick recovery) while a "chronic" response can be defined as taking up to 36 hours to initiate and recover at >96 hours (slow response, slow recovery). If one had a panel with five biomarkers placed on it, at any given time, some would be informative and some would not. More importantly, the expression pattern of the biomarker panel will allow one to determine the temporal and dose aspects of the exposure. It has been proposed that testing be performed every 24 hours in deployed conditions in locations with unknown toxins. If testing occurs 9 hours after an exposure (Urine test #1):

Urine Test #1
BM1   Slight increase, but barely within detection limits
BM2   Big increase and very detectible
BM3   no change from control
BM4   no change from control
BM5   Slight increase, but barely within detection limits.

So in Test #1, only one biomarker was informative that decrement might have taken place. This is why both a panel of markers consisting of several 'types' of biomarker responses are needed. If this individual retests after another 24 hours:

Urine Test #3
BM1   no change from control
BM2   no change from control
BM3   no change from control
BM4   Big increase and very detectible
BM5   Big increase and very detectible

This test will confirm an exposure by seeing the increases in biomarker 4 and 5. Notice that some markers were informative, some were not, and this will differ depending upon the time tested. Additionally although BM3 is a good biomarker, it did not provide information in this example. It could be argued that Test #3 would also provide information that an exposure initiating renal injury has taken place, but it would not provide any idea of the time of exposure, which could be an advantage. At some point, with additional research and discovery, the level of toxin exposure may be monitored by total biomarker concentration as well as toxin identity.
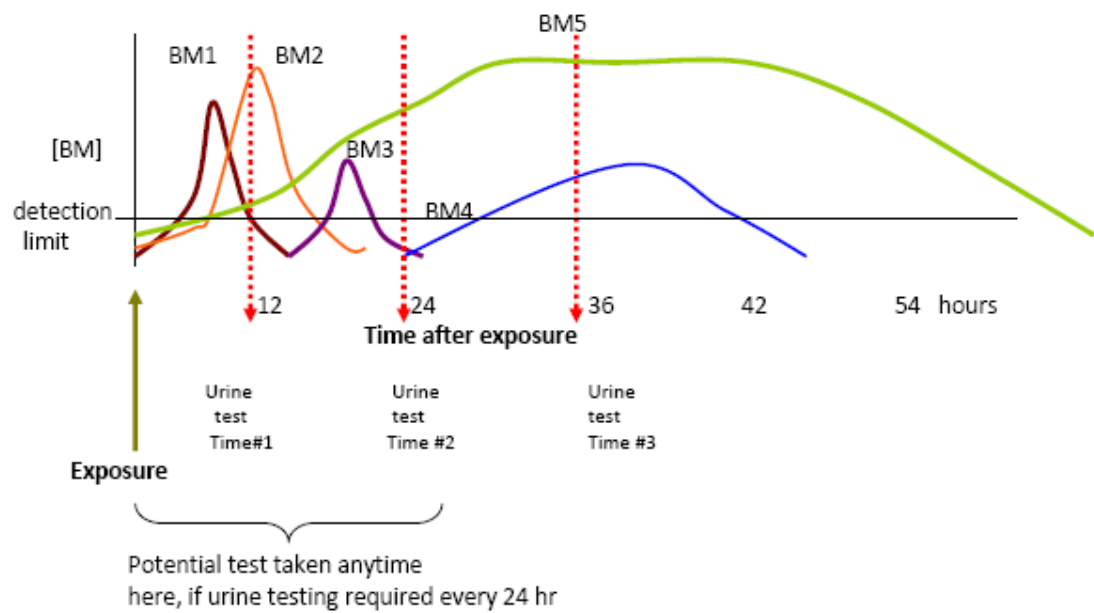
Figure 94: Types of biomarker responses for monitoring
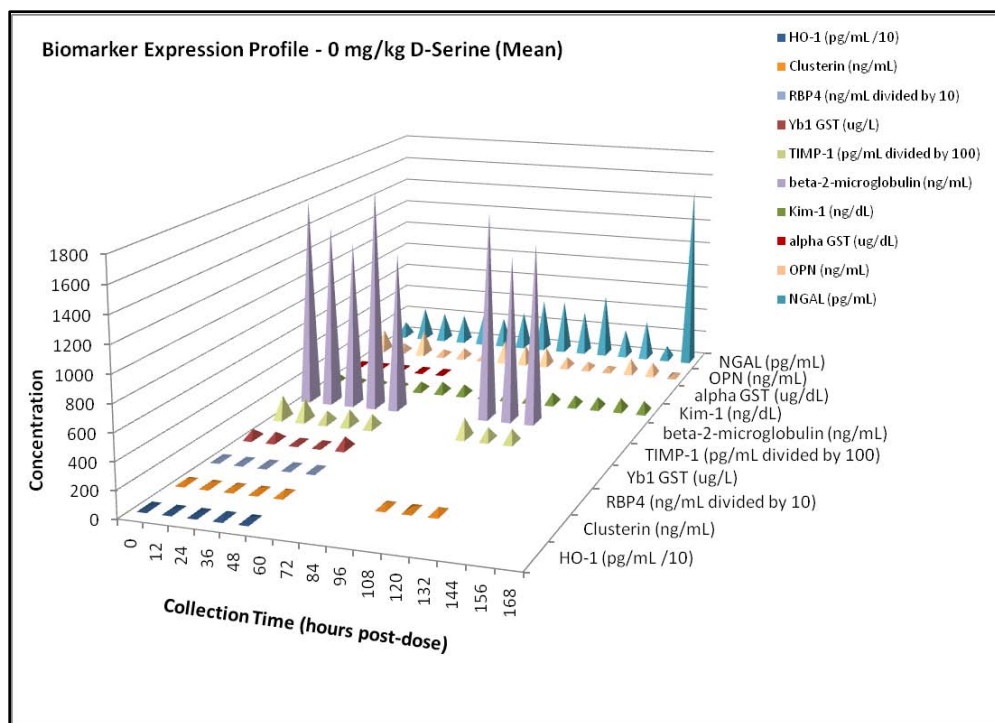
Figure 95: Urinary biomarker panel response at control (baseline) levels
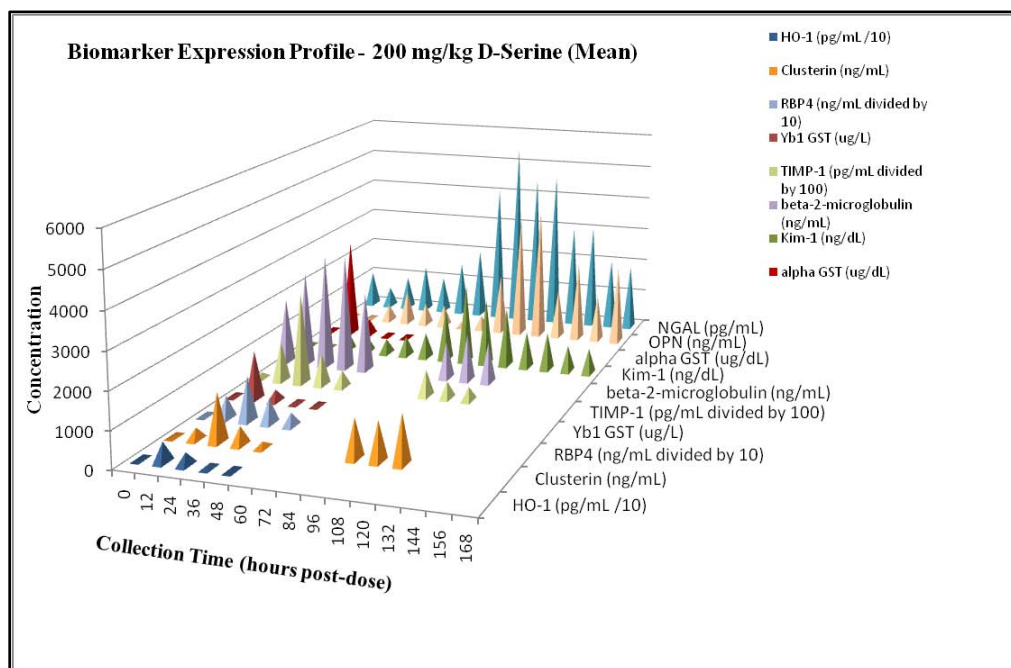*Note differences in marker concentration units.*

Figure 96: Urinary biomarker panel response at 200 mg/kg D-serine exposures
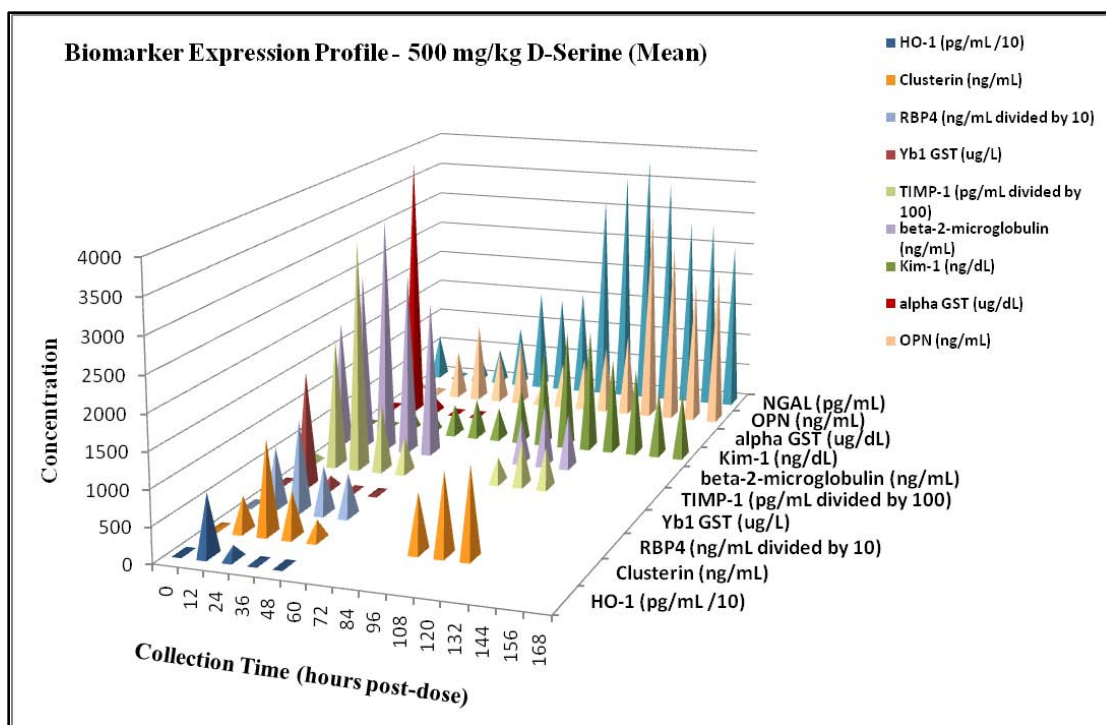*Note differences in marker concentration units.*

Figure 97: Urinary biomarker panel response at 500 mg/kg D-serine exposures.
Note differences in marker concentration units.

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185

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6. LIST OF SYMBOLS, ABBREVIATIONS, AND ACRONYMS

AKI – acute kidney injury
ANOVA – analysis of variance
AP – alkaline phosphatase
BACC – baseline-adjusted creatinine concentration
BUN – blood urea nitrogen
CKD – chronic kidney disease
CREA – creatinine
CV – coefficient of variation
EDTA – ethylenediaminetetraacetic acid
ELISA – enzyme linked immunosorbant assay
HRP – horse radish peroxidase
LLOD – lower limit of detection
LLOQ – lower limit of quantitation
OD – optical density
QC – quality control
RHBP – Biotechnology Branch, Biosciences and Protection Division
RSM – Reference Standard Material
TBIL – total bilirubin
TMB – Tetramethyl benzidine